# The link between multidrug resistance and oxidative stress

Yiakouvaki, Anthie

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# The link between multidrug resistance and oxidative stress

Submitted by Anthie Yiakouvaki

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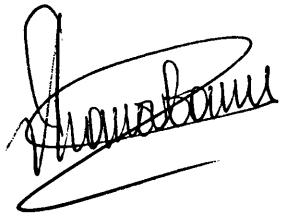
University of Bath
Department of Pharmacy and Pharmacology
Claverton Down
Bath BA2 7AY







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# ABSTRACT

Much evidence has been accumulated that suggest a link between the phenomenon of multidrug resistance (MDR) and the resistance to oxidative stress. Most of these studies have been performed in tumour cells exhibiting MDR phenotype and usually demonstrate that the development of MDR renders the tumour cells resistant to oxidizing agents such as hydrogen peroxide ($H_2O_2$) or ultraviolet A (UVA, 320-400nm). However to date, no study have revealed the underlying mechanism involved. In this thesis, we used a cell model composed of two human Jurkat T cell lines of parental and $H_2O_2$ resistant to assess the pathways behind cellular resistance to oxidative stress and the possible link between the development of MDR phenotype and resistance of cells to the oxidising agents.

To this regard, both parental and $H_2O_2$ resistant cells were first characterised in terms of the level of susceptibility to oxidizing agents $H_2O_2$ and UVA and then the involvement of intracellular antioxidant defence and labile iron was investigated. Resistance of cells to $H_2O_2$ was confirmed and our data revealed that necrosis is the primary mode of cell death induced by both $H_2O_2$ and UVA. The characterisation of antioxidant defence mechanism in these cell lines revealed that resistance of cells to $H_2O_2$ is mainly due to higher glutathione peroxidase (GPx) activity. Furthermore both higher intracellular levels of glutathione (GSH), ferritin (Ft) and heme-oxygenase 1 (HO-1) appeared to contribute to higher $H_2O_2$ resistance of $H_2O_2$ resistant Jurkat cells.

Since exposure of skin cells to UVA or $H_2O_2$ has been shown to cause an immediate increase in intracellular labile iron pool (LIP), we sought to monitor both the 'basal' and 'oxidant-induced' increase in LIP following exposure of cell lines to both UVA and $H_2O_2$ insults. The results revealed that although the 'basal' level of LIP in both parental and $H_2O_2$ resistant cell lines was similar, the $H_2O_2$-induced level of labile iron release was much higher in parental cells than in $H_2O_2$ resistant cell line. This finding strongly suggested that cellular resistance to $H_2O_2$ is tightly associated to intracellular level of LIP and that the "induced" rather than the "basal" level of labile iron is responsible for the increased susceptibility of cells to oxidative stress.

Interestingly, the exposure of both parental and $H_2O_2$ resistant cells to UVA promoted both similar level of labile iron releases and percentage survival consistent with the notion that $H_2O_2$ and UVA should exert their effect on cells via different oxidant mechanism.

Subsequently, an attempt was made to correlate the level of $H_2O_2$-induced iron release and the extent of cell damage following oxidative treatment. For this purpose, cells were treated either with Desferal (DFO) or hemin (iron loading) prior to a range of doses of $H_2O_2$. The DFO treatment of normal and $H_2O_2$ resistant cells completely abolished both the 'basal' and 'induced' level of LIP and protected cells against $H_2O_2$-mediated damage. Interestingly hemin pre-treatment dramatically decreased the $H_2O_2$-induced level of labile iron release in the parental cells, while increasing the level of $H_2O_2$-induced labile iron release in $H_2O_2$ resistant cells. Furthermore, hemin pre-treatment protected the parental cells against $H_2O_2$-induced necrotic cell death, but sensitises $H_2O_2$ resistant cells to the cytotoxic effect of $H_2O_2$. The differential response of hemin treatment in both parental and $H_2O_2$ resistant cells highlighted the crucial importance of oxidant-induced labile iron release in the increased susceptibility of cells to oxidative damage.

Finally, we could not establish any link between MDR and resistance to $H_2O_2$, since both parental and $H_2O_2$ resistant cells appeared to have similar drug efflux activity of PgP function and no genes responsible for the MDR phenotype (mdr-1, mrp and mxr) was detected in either cell line.

In summary these data provide the first direct evidence for association of intracellular level of LIP and the resistance of cells to oxidative damage and suggest pathways for protection through iron chelation.

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| **ATP** | Adenosine 5'-triphosphate |
| **AIF** | Apoptosis inducing factor |
| **Apaf-1** | Apoptotic protease activating factor-1 |
| **BSA** | Bovine serum albumin |
| **BSO** | Buthionine S-R Sulfoximine |
| **CA** | Calcein |
| **CA-AM** | Calcein-acetoxymethyl ester |
| **CA-assay** | Fluorescence calcein assay |
| **CA-Fe** | Calcein bound iron |
| **Caspases** | Cysteine aspartate-specific proteases |
| **CDNA** | Complementary deoxyribonucleic acid |
| **CO** | Carbon monoxide |
| **$\Delta\Psi m$** | Mitochondrial membrane potential |
| **DFO** | Desferrioxamine or desferrioxamine mesylate or desferal |
| **DMSO** | Dimethyl sulphoxide |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylene-diaminetetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EMEM** | Earle's modified minimum essential medium |
| **FAS** | Ferrous ammonium sulphate |
| **FCS** | Fetal calf serum |
| **$Fe^{2+}$** | Ferrous iron |
| **$Fe^{3+}$** | Ferric iron |
| **FITC** | Fluorescein Isothiocyanate |
| **FL1** | Fluorescent intensity 1 |
| **Ft** | Ferritin |

| | |
|---|---|
| **GPx** | Glutathione peroxidase |
| **GSH** | Glutathione (reduced) |
| **GSSG** | Glutathione disulhide (oxidised glutathione) |
| **GR** | Glutathione reductase |
| **Hb** | Haemoglobin |
| **HNE** | 4-hydroxy-2-trans-nonenal |
| **HO** | Heme oxygenase |
| **HO-1** | Heme oxygenase 1 |
| **$H_2O_2$** | Hydrogen peroxide |
| **$kJ/m^2$** | kiloJoules per meter square |
| **IRE** | Iron response element |
| **IRP** | Iron regulatory protein |
| **LIP** | Labile iron pool |
| **$LO^{\bullet}$** | Lipid alkoxyl radical |
| **$LOO^{\bullet}$** | Lipid peroxyl radical |
| **LOOH** | Lipid hydroperoide |
| **MDR** | Multidrug resistance |
| **MRP** | Multidrug resistance associated protein |
| **MTT** | 3-(4,5-dimethylthiazol-2-ye)-2,5-diphenyltetrazolium bromide |
| **MXR** | Mitoxatrone |
| **NADH** | Nicotine adenine dinucleotide |
| **NADP** | Nicotine adeninedinucleotide phosphate |
| **NO** | Nitric oxide |
| **$^1O_2$** | Singlet oxygen |
| **$O_2^{\bullet -}$** | Superoxide anion |
| **$OH^{\bullet}$** | Hydroxyl radical |
| **PBS** | Phosphate buffered saline |
| **PgP** | P glycoprotein P |
| **PTP** | Permeability transition pore |
| **RNA** | Ribonucleic acid |
| **ROS** | Reactive oxygen species |

| | |
|---|---|
| **RT** | Room temperature |
| **SDS** | Sodium dodecyl sulphate |
| **SFM** | Serum free media |
| **SIH** | Salicyladeldehyde isonicotinoyl hydrazone |
| **SOD** | Superoxide dismutase |
| **Tf** | Transferrin |
| **TfR** | Transferrin receptor |
| **UVA** | Ultraviolet A |
| **UVB** | Ultraviolet B |
| **UVC** | Ultraviolet C |
| **w/v** | Weight per volume |

# CHAPTER 1

# INTODUCTION

## 1.1 Oxidative stress

It has been recognised more than a hundred years ago that oxygen ($O_2$), the breath of life, apart from being beneficial can be also toxic to all living organisms. Except for certain anaerobic and aerotolerant unicellular organisms, $O_2$ is required for efficient production of energy by the use of $O_2$-dependent electron transport chains. However, when $O_2$ is supplied at concentrations greater than normal it can be toxic to all living organisms. Nowadays, it is known that both beneficial and damaging effects of $O_2$, are caused by the same types of reactive oxygen species (ROS).

Reactive oxygen species is a collective term that includes not only the oxygen radicals (i.e. superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$)) but also some non-radical derivatives such as singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$) and ozone ($O_3$), which are capable of forming radicals. An elevation in the cell by a steady state concentration of a variety of ROS results in an imbalance between the mechanisms triggering oxidative conditions and the cellular anti-oxidant defences. This characterizes a phenomenon termed "oxidative stress". Potentially dangerous oxygen-linked damaging processes are thought to form the basis of a number of physiological and pathophysiological events

such as inflammation, ageing, carcinogenesis, drug action, drug toxicity and more recently programmed cell death.

### *1.1.1* Reactive oxygen species (ROS)

Both exogenous (i.e. xenobiotics, ozone, photochemical smog, ultraviolet light, ionising radiation), as well as endogenous (i.e. mitochondrial respiration, phagocytic oxidative burst) processes can generate highly reactive compounds known as radicals. Here discussion will be focused on the exogenous processes that can generate ROS and in particular ultraviolet A (UVA) and $H_2O_2$.

A free radical is defined as "atom or molecule with one or more unpaired electrons in an orbital in the outermost electron shell". Free radicals are capable of independent existence and are able to donate or take an electron from an unpaired electron to another molecule, generating another radical by chain reaction, which enhances the initial damage. The primary target of free radicals is the lipid bilayer of the membrane. However, free radicals can also oxidize protein, lipid and carbohydrate.

Molecular $O_2$ has two unpaired electrons in a parallel spin and can be a powerful oxidizing agent, since it can easily absorb electrons from surrounding molecules. However, if $O_2$ attempts to oxidize another atom or molecule by accepting a pair of electrons from it, both of these electrons would be of antiparallel spin and according to Pauli's principle wouldn't fit in the $\pi^*$ orbitals of $O_2$. This imposes a restriction on electron transfer, which tends to make $O_2$ accept its electrons one at a time. For example, reduction of $O_2$ to water ($H_2O$) requires a series of four one-electron-uptake steps, which

involves a series of ROS, as shown in **Figure 1.1**. This process makes $O_2$ react with biomolecules poorly, unless a transition metal such as iron is present as a catalyst.

$$O_2 + e^- \longrightarrow O_2^{\bullet -}$$

$$O_2^{\bullet -} + e^- + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + e^- + H^+ \longrightarrow H_2O + OH^\bullet$$

$$OH^\bullet + e^- + H^+ \longrightarrow H_2O$$

***Figure 1.1***: The series of ROS emerging from the step by step reduction of $O_2$ to $H_2O$, involving 4-electron ($e^-$) reduction.

The first product of an electron reduction of $O_2$ molecule yields the $O_2^{\bullet -}$ . Production of $O_2^{\bullet -}$ seems to occur within all aerobic cells and more specific in the endoplasmic reticulum and during respiration (electron transport chains of mitochondria). Microsomal fractions containing endoplasmic reticulum have been shown to produce $O_2^{\bullet -}$ via NADPH oxidase enzymatic system. At physiological $O_2$ levels, it has been suggested that about 1-3% of the $O_2$ reduced in mitochondria may form $O_2^{\bullet -}$, depending on intra-mitochondrial $O_2$ concentrations. Superoxide anion is not readily reactive towards most biomolecules, including lipids and nucleic acids (Fridovich, 1978). However, it may react with certain proteins and inactivate them, especially proteins in the presence of transition metals prosthetic groups such as heme moieties or iron-sulphur clusters (Gardner *et al*, 1995). As a consequence, $O_2^{\bullet -}$ toxicity is highly dependent on the availability of iron in the system.

The most important reaction of $O_2^{\bullet -}$ is the dismutation reaction which produces the well known oxidant $H_2O_2$. This reaction can occur either spontaneously or via catalysis

by a group of enzymes, the superoxide dismutases (SOD). $H_2O_2$ is only a weak oxidizing and reducing agent and is generally poorly reactive, but in the presence of transition metals, it is capable of inactivating proteins via oxidation of essential thiol (-SH) groups or proteins containing iron-sulphur (Fe-S) clusters, reduced heme moieties or copper prosthetic groups. Most of the damaging effects of $H_2O_2$ are thought to be the result of the formation of $OH^{\bullet}$ (see also section 1.2.2). Unlike $O_2^{\bullet -}$ , $H_2O_2$ crosses membranes readily and can therefore migrates within the cell and extends cellular damage. The biological importance of $H_2O_2$ also arises from its ability to diffuse through hydrophobic membranes. Hydrogen peroxide has also been found to be generated after UVA radiation (see section 1.3).

Hydroxyl radical ($OH^{\bullet}$) is the most reactive of all oxygen radicals and will readily oxidize lipids, proteins, carbohydrates and nucleic acids. The importance of $OH^{\bullet}$ as an oxidant in biological systems was first suggested when it was shown to be generated by X-ray irradiation. The $OH^{\bullet}$ is the product of Fenton reaction involving $H_2O_2$ and reduced iron (see also section 1.2.2).

*1.1.2* Iron and reactive oxygen species (ROS)

The cytotoxic actions of ROS are increased in the presence of a transition metal catalyst, with iron being the most abundant transition metal. The ability of this transition metal to exist in two redox states makes it useful at the catalytic centre of fundamental biochemical reactions. Iron can react with $O_2^{\bullet -}$ and $H_2O_2$, giving rise to a highly reactive $OH^{\bullet}$ radical via the Haber-Weiss or Fenton reaction (Aust *et al*, 1985, Halliwell and Gutteridge 1999), respectively. The Haber-Weiss reaction is shown below:

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe} O_2 + H_2O + OH^{\bullet}$$

The Fenton reaction is usually written:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

Fenton chemistry is a prime example of a damaging free radical reaction that is catalysed by iron ($Fe^{2+}$) and the best characterised is the generating $OH^{\bullet}$.

However, in cells the true catalyst of Fenton reaction is more likely to be the low-molecular mass (LMrFe) free iron pool or iron made available from other cellular sources under conditions of oxidative stress. This pool provides the cell with a relatively accessible form of iron for incorporation into cytosolic enzymes and proteins. When present in extracellular compartments has been found to be associated with ligands such as citrate, acetate, and phosphate, and also with ATP, GTP, pyrophosphates and amino acids (reviewed by Symons and Gutteridge, 1998). The amount of iron within this pool is dependent on the type of cell, with values ranging from 0.2 to 1.5 μM (Epsztejn *et al*, 1997) and is probably present in the ferrous state. The iron which can be extracted from proteins by ligands into LMrFe form has been termed the "labile iron pool" (LIP) by Jacobs in 1977 and White in 1976 (see section 1.2.2).

## 1.2 Iron

### *1.2.1* General aspects

Elemental iron is essential for normal cell growth and proliferation, and is required by almost every organism. Living organisms, from bacteria to mammals, appear to have

selected iron for achieving a large number of essential biological processes in which is involved as part of or as a metal cofactor for many proteins and enzymes, either nonheme or hemoproteins (Aisen and Listowsky, 1980).

In the adult human body the largest pool of iron (about 75%) is present in heme, mainly found in hemoglobin and myoglobin, which transport oxygen. Most $O_2$ carried in the blood is transported by hemoglobin. The hemoglobin molecule has four protein subunits, two α-chains and two β-chains. Each chain carries a heme group with iron in the $Fe^{2+}$ state. Only the ferrous iron form ($Fe^{2+}$) of hemoglobin can bind $O_2$, permitting efficient energy production and allowing the operation of enzymes that catalyse metabolic transformations. Heme can be synthesised in mammalian cells and by many bacteria. **Figure 1.2** illustrates a diagrammatic representation of the heme synthesis pathway. The heme biosynthetic pathway is probably identical in all mammalian cells and involves eight enzymes, four of which are cytoplasmic and four of which are localised in the mitochondria. In brief, heme synthesis is mainly catalysed by the enzymes coporphyrinogen oxidase (in the cytosol) and protoporphyrinogen oxidase (in the mitochondria). The first step occurs in the mitochondria and involves the condensation of succinyl CoA and glycine to form 5-aminolevulinic acid (ALA). The next four steps take place in the cytosol. The final three steps occur in the mitochondria and involve the insertion of ferrous iron into photoporphyrin IX by ferrochelatase and finally the production of heme (reviewed by Ponka, 1997). Heme biosynthesis is normally remarkably efficient.

Besides hemoglobin, other hemoproteins include: (a) cytochromes, which are present in mitochondria and required for the electron chain reactions; (b) catalase and peroxidase,

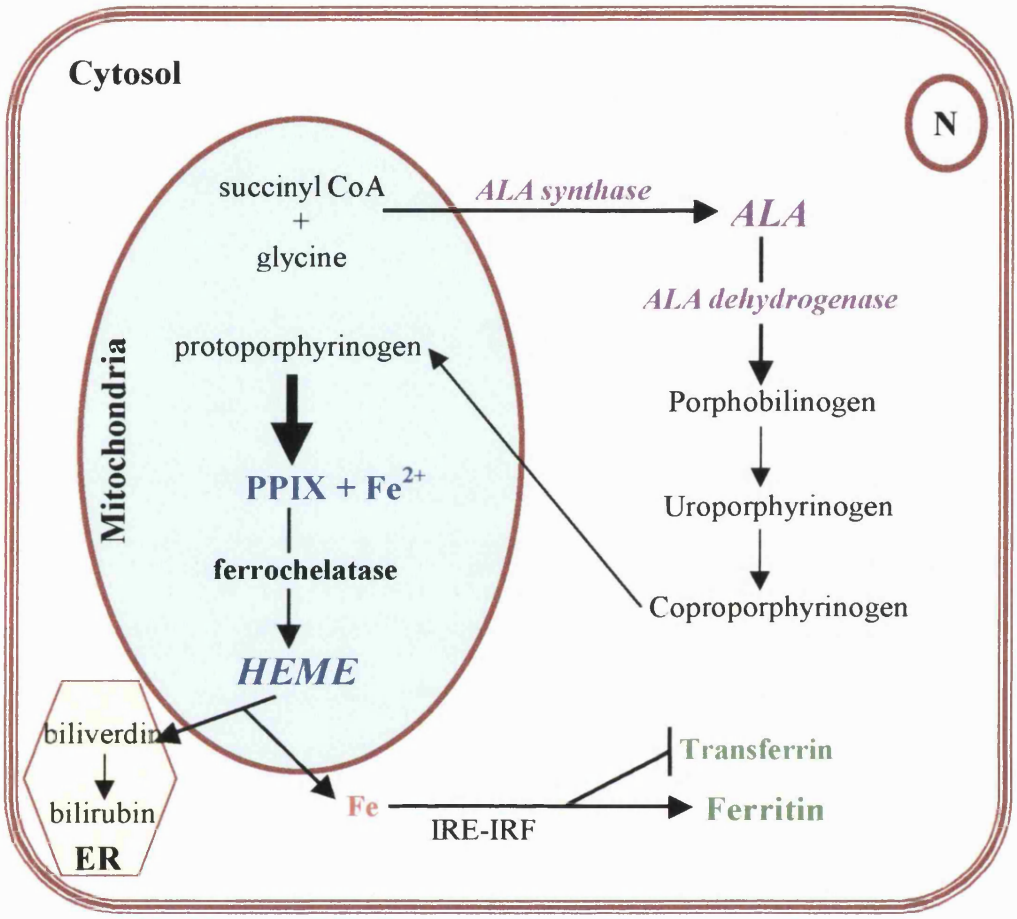



***Figure1.2:*** Diagrammatic representation of heme synthesis in mammalian cells.

which react with $H_2O_2$ and protect against uncontrolled cellular oxidation (section 1.6); and (c) tryptophan oxygenase, which is used for intermediary metabolism and catalyses conversion of tryptophan to N-formylkymeremine, using $O_2$ as the oxidant (section 1.4).

Around 10% of the total iron is required for numerous metalloenzymes and 15% is present in the iron-storage protein, ferritin (Ft). These iron-complexing molecules leave body fluids and cells with extremely low concentration of "free" transit iron. Nevertheless there is now strong evidence of a pool of catalytically active "free" iron known as labile iron pool (LIP). The LIP exists within cells at a concentration of 0.1-1 μM and is accessible to permeant chelators. This pool of reactive iron is known to be potentially harmful, since it can catalyse the formation of ROS such as $OH^{\bullet}$ and lipid-derived alkoxyl and peroxyl radicals (see section 1.2.2). Therefore, balancing intracellular iron is essential for survival of the cells (see section 1.3).

### *1.2.2* Labile iron pool (LIP)

The metabolically active forms of intracellular iron are components of cytosolic LIP, also referred to as chelatable iron pool (Jacobs, 1977). It contains the metabolically and catalytically reactive iron of the cells. Labile iron pool is associated with important functions: (a) physiologically, as readily available sources of iron for incorporation into proteins; (b) pharmacologically, as targets for chelators or metal scavengers; and (c) toxicologically, as vehicles for promoting the formation of free radicals.

The LIP provides the cell with a relatively accessible form of iron for incorporation into cytosolic enzymes or proteins (Breuer *et al*, 1996). Although, the nature of the LIP is not fully characterised, there are several observations (see Harrison and Arosio, 1996),

suggesting that it consists of both ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) ligands. Labile iron pool exists in dynamic equilibrium as $Fe^{2+}$ and $Fe^{3+}$ forms, which is mainly controlled within ferritin (Ft) where $Fe^{3+}$ is deposited after oxidation of the $Fe^{2+}$ form.

Unlike Ft bound iron, the LIP is loosely bound to macromolecular complexes and they are sensed by the cytosolic iron regulatory proteins (IRPs). The LIP is maintained by a balanced movement of iron from extracellular and intracellular sources, which can be increased by endocytosis of transferrin (Tf) bound iron or non-Tf bound iron and degradation of Ft (see section 1.3).

In general, all aspects of intracellular homeostasis are monitored by the LIP, since it is rapidly transmitted through the cell and constitutes a real turning point of metabolic pathways of iron-containing compounds.

In contrast to the intracellular LIP, there is also the presence of the extracellular LIP, which is often associated with pathological conditions. This form of LIP has been originally observed in iron-over-loaded thalassemia patients whose plasma Tf iron-binding capacity was surpassed (Hershko *et al*, 1978). In addition, to thalassemia, other conditions of iron imbalance have been defined (i.e. hemochromatosis (HH)), in which the LIP has been found to be bound to ligands other than Tf or non-Tf bound iron. Finally there is evidence for age-related accumulation of LIP associated with rheumatoid arthritis (Guillen *et al*, 1998).

Primarily, attempts to quantify and assess the LIP relied on methods that involved homogenisation of the cells and tissue, and iron content was then determined using electron paramagnetic resonance with iron chelators (Kozlov *et* al, 1992) or DFO-chelatable $^{59}Fe$ (Rothman *et al*, 1992). However, cell or tissue homogenisation required

cell disruption that could alter the equilibrium between free and bound iron as well as its oxidation state. Therefore, detection of LIP has necessitated the development of distinct probes.

Among the different approaches developed for analysing LIP, those based on the selective interaction of LIP with iron chelators provide the simplest and most accessible tools. Indeed, fluorescence detection of iron on probes or metal-sensing molecules that undergo swift changes in signal properties as a result of an interaction with labile iron has been a favoured approach for the estimation of LIP. Cabantchick and co-workers in 1997 have developed one of the most efficient fluorescent assays, called the fluorescent calcein assay (CA-assay) (see section 2.7). Calcein is a fluorescent probe that binds readily, stoichiometrically (1:1) and reversibly with a fraction of $Fe^{2+}$ associated with LIP, while forming fluorescent quenched CA-bound iron complexes (CA-Fe). Consequently, the quenched fluorescence of the CA-Fe complex in solution can be fully restored by addition of excess chelators such as salicyladedehyde isonicotinoyl hydrazone (SIH) which can abstract iron from CA. Thus, the CA fluorescence assay is based on the availability of fast permeating high affinity chelators, with SIH being the best candidate since it can chelate $Fe^{2+}$ and $Fe^{3+}$ forms of iron and has a binding constant $10^{29}$ $M^{-1}$ for $Fe^{3+}$ and $10^{20}$ $M^{-1}$ for $Fe^{2+}$ (Tsakack *et al*, 1996 and Epsztejn *et al*, 1997). After the development of this assay, a number of different studies were carried out to assess changes in the LIP after various treatments.

## 1.3 Iron Homeostasis

Mammalian cells maintain constant levels of metabolically active iron through the regulation of iron uptake and storage. The pathway of iron uptake via transferrin receptor (TfR) and the iron storage in Ft are co-ordinately regulated at the post-transcriptional level by cytoplasmic factors known as IRPs. These regulatory mechanisms operate in order to prevent the expansion of the intracellular LIP, but still secure adequate supply of iron for the synthesis of iron-dependent proteins. Although, there is a widespread agreement that these regulatory mechanisms are utilised in many cell types, additional regulatory pathways may operate in erythroid cells due to their specialised function in hemoglobin synthesis (see section *1.3.3*).

### *1.3.1* Transferrin (Tf)

Transferrin belongs to a family of related-binding proteins that includes:(a) lactoferrin, which is found both intracellularly and in secretions such as milk, tears and semen; (b) ovotransferrin, which is present in egg white and (c) melanotransferrin, which is formally known as tumour antigen p97. These proteins share a high degree of sequence homology and their molecular weight is around 80 kDa. Transferrin is the plasma iron-binding glycoprotein and functions as the major vehicle for transfer of iron in the body between sites of absorption, storage and use. It is normally the only source of iron for hemoglobin synthesis. Transferrin has a high affinity for $Fe^{3+}$ ($K_d = 10^{-23}$ mol/L) and only one $Fe^{3+}$ attaches to each of one of the two globular domains extremely tight at physiological pH. Transferrin exists as a mixture of iron-free (apoTf), one iron (monoTf) and two iron (difericTf) forms of the molecule. The primary function of Tf is to accept

iron from plasma and to transport iron into various cells and tissues, by binding to membrane receptors (Tf-receptors, see below), internalise iron via endocytosis and finally release its iron at acidic pH in late endosomes (**Figure 1.3**).

Transferrin receptors (TfRs) provide for controlled access of Tf to the cells. There are two forms of TfR, TfR1 and TfR2, which have distinct cell- and tissue- specific pattern. However, TfR2 has only been described in 1999 in liver, liver-derived and human erythroleukemia K562 cell lines, so TfR1 has been the most studied one and was simply designated the TfR. Transferrin receptor is expressed in all cell types apart from mature erythrocytes and other terminally differentiated cells. It is comprised of two disulfide-bonded identical 90 kDa subunits and is far more abundant than TfR2. Transferrin binds to the TfR at the cell surface and is internalised through clathrin-coated pits into endosomes (**Figure 1.3**). At the acidic pH of the endosome, iron dissociates from Tf and is taken to the cytoplasm via membrane transporters (i.e. DMT-1 and H-ATPase). Once the iron has passed through the membrane it enters the intracellular LIP compartment. The iron now inside the cell can be used for metabolic functioning (modulation of the IRPs activity) can be taken up by mitochondria or can be stored in Ft (**Figure 1.3**). After the return of the receptor to the cell surface, the extracellular pH triggers the release of apo-Tf, allowing another round of binding and endocytosis to begin. Thus Tf may protect cells against oxidative damage by binding to iron and preventing oxidative reactions catalysed by iron (Klausner *et al*, 1993; Kuhn, 1994; Guo *et al*, 1995; Piccard *et al*, 1998).

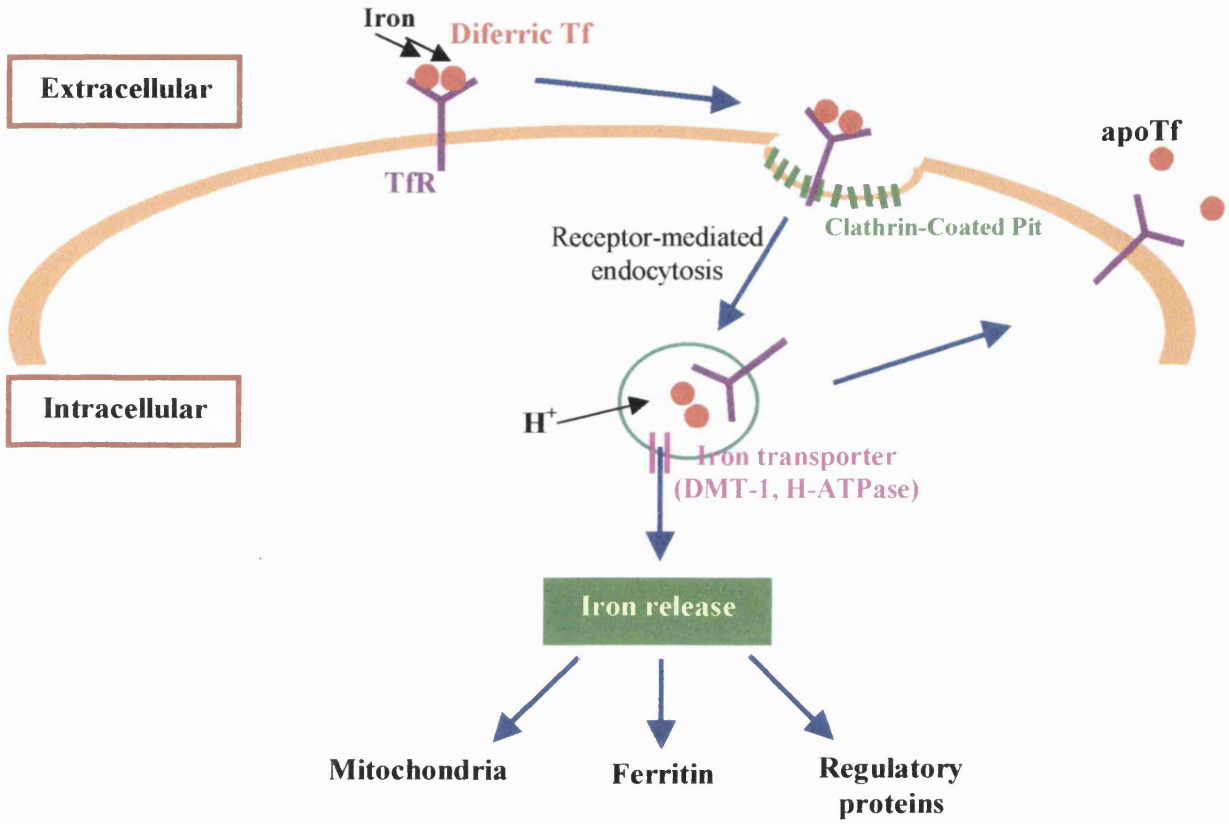



***Figure 1.3*:** Diagrammatic representation of the uptake of transferrin-mediated iron transport into cells (Adapted from Aisen *et al*, 2001).

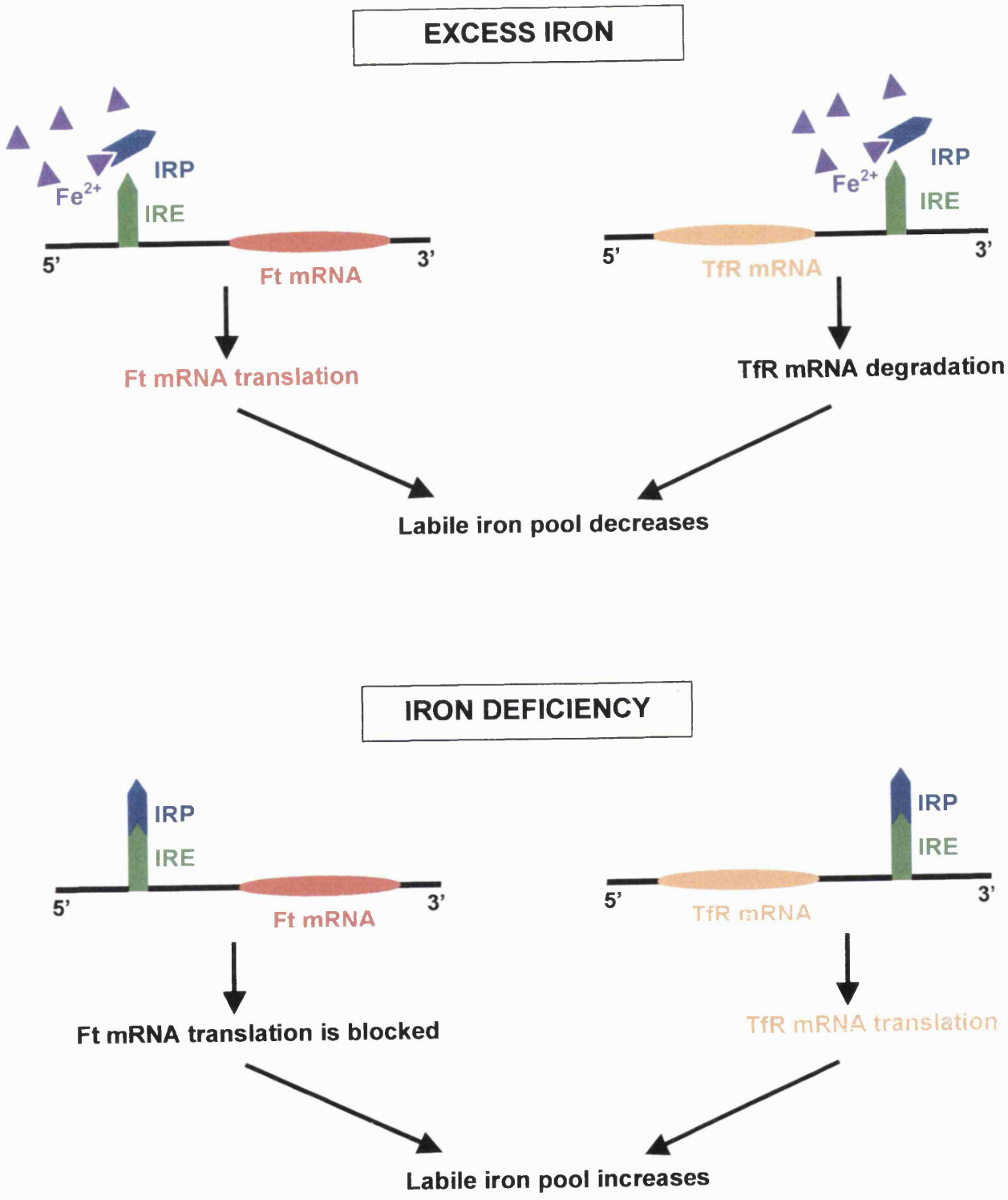
*1.3.2* Iron regulatory proteins (IRPs)

The level of LIP is thought to be both sensed and homeostatically controlled by IRP-1 and IRP-2, which function as regulators of iron uptake and distribution processes within cells, and co-ordinately regulate the expression of the transferrin receptor (TfR) and of Ft. Iron regulatory protein-1 resembles mitochondrial aconitase in sequence, it can assemble an iron-sulfur cluster and is enzymatically active (Kaptain *et al*, 1991). So, activation of IRP-1 involves an iron-sulphur cluster, which depending on its iron content, acts either as an RNA-binding protein or as a cytoplasmic aconitase (Kuhn, 1994). For example:

$$(3Fe-4S)^{+} + Fe^{2+} \longrightarrow (4Fe-4S)^{2+}$$

Unlike IRP1, IRP2 does not show any aconitase activity, nor does it accumulate an iron-sulphur cluster. It appears to be regulated by proteasome-mediated degradation.

Indeed, the stem-loop structures, called iron responsive elements (IREs), which are present in the respective mRNAs of genes of the iron-storage (Ft) and iron-uptake (TfR) proteins, interact with the cytosolic IRPs and regulate the level of LIP in the cells. In particular, an increase in reactive labile iron in the cells will cause inactivation of IRP-1 (90 kDa) and degradation of IRP-2 (105 kDa), leading to the induction of Ft mRNA translation and degradation of TfR mRNA. The resulting *de novo* synthesised Ft protein will sequester the excess iron resulting in a decreased level of intracellular labile iron (**Figure 1.4**). In contrast, an iron deficiency leads to the binding of IRPs to IREs resulting to inhibition of Ft mRNA translation and induction of TfR protein synthesis (**Figure 1.4**). Unlike TfR1, TfR2 lacks an IRE, so its expression is insensitive to iron status. It has been




*igure 1.4*: Diagrammatic representation of the coordinated regulation of iron uptake and storage at posttranslational vel (Adapted from Henderson *et al*, 1996).

suggested that TfR2 is most probably regulated by the cell cycle accommodating the needs of cell proliferation, since it is over-expressed in the iron-overloaded liver of HH (Fleming *et al*, 2000 and Kawabata *et al*, 2000).

The action of IRP proteins can be further modulated through the activation of signal transduction cascades, like protein kinase C (PKC) which when activated phosphorylates either IRP1 or IRP2 through different serine residues and increases IRP1/IRP2 binding to the IRE (Schalinske and Eisenstein, 1996).

Other proteins that process stem-loop structures either on the 5' or 3' untranslated portion of their mRNA include erythroid 5-amino levulinic acid synthase (ALA-synthase, see section 1.2.1) which is involved in heme biosynthesis (Gardner *et al*, 1991), mitochondrial aconitase (Dandekar *et al*, 1991) and DMT-1 (reviewed by Sheth and Britternham, 2000). Additional IRE sequences have also been identified in ferroportin1 (Donovan *et al*, 2000) and IREG1 (McKie *et al*, 2000) which plays a role in iron efflux across membranes to plasma but their function in IRP binding has not yet been determined.

An important finding correlating IRP and 5-ALA was also observed by Pourzand *et al* in 1999a. They demonstrated that there is a strict dependence on enhancement on LIP levels by photoporphyrin IX (PPIX) and the level of IRP activation. They proposed that the level of IRP activation could serve as a better marker for iron deficiencies than TfR expression since is directly correlated with the level of intracellular LIP.

Finally, relative ratios of IRP1/IRP2 differ between tissues with IRP1 being the highest in liver, kidney, intestine and brain, and the least abundant in pituitary and pro-B-lymphocytic cell lines (Thomson *et al*, 1999).

*1.3.3* Ferritin (Ft)

An additional mode of regulation of LIP operates through the Ft molecule. It is generally accepted that increased cellular iron levels appear initially in the LIP, but the excess iron is sequestered into Ft molecules (Piccard *et al*, 1998).

Indeed, most of the iron that is not metabolised is stored in Ft. Ferritin is the major iron storage protein. It is ubiquitous in mammalian cells and is tightly regulated by IRPs. Holoferritin can accommodate approximately 4,500 atoms of iron, doubling its molecular mass to 900 kDa. This protein forms a hetero-oligomeric protein shell composed of a light- (L, 19kDa) and heavy- (H, 21kDa) chain subunits (Munro and Linder, 1978; Theil, 1987). The heavy chain of Ft has ferroxidase activity, which oxidises $Fe^{2+}$ to $Fe^{3+}$ for iron uptake by the Ft molecule. The H-Ft has a high affinity for $Fe^{3+}$ ($K_d = 10^{-25}$ mol/L). Iron storage in the ferric form ($Fe^{3+}$) protects iron from reduction (Balla *et al*, 1992). In contrast, the L-Ft chain has no ferroxidase activity, but has a nucleation site that facilitates iron core formation within the cell and has more iron storage capacity. The H-to-L ratio is not fixed but is readily modified in response to different stimuli such as inflammation and stress, promoting the cell to adjust to changes in iron metabolism. For example, an increase in the proportion of the L-Ft is associated with iron storage, whereas the H-Ft is more abundant when iron is needed for cellular metabolism. The role of iron in controlling the amount of Ft in mammalian cells is mostly via the regulation of Ft synthesis at a translation step (section 1.5.2).

Small quantities of Ft are also present in human serum and are elevated in conditions of iron overload and inflammation (Torti and Torti, 1994). It is generally believed that serum Ft has a low iron content (roughly each μg/L Ft corresponds to 8mg storage iron)

even in iron over-loaded individuals (Linder *et al*, 1996). In 1995, Beaumont and co-workers identified a new genetic disorder in which patients with increased levels of serum Ft present early onset cataract. This genetic syndrome is due to mutation on the L-Ft gene located on chromosome 19q13.3 and is believed that cataract is a consequence of excessive L-Ft production. However, the exact mechanism underlying the latter has not yet been identified. Moreover, iron overloaded cells, in conditions such as HH, may contain another storage form of iron called hemosiderin.

Hemosiderin is a degradation product of Ft since under conditions of iron excess, Ft is taken-up by lysosomes where it undergoes a partial dissolution of the core resulting in the formation of insoluble hemosiderin (Harrison and Arosio, 1996). Hemosiderin is a lipoprotein found in lysosomes, which appears to be a dead end in the metabolism of iron and Ft.

Recently, Levi *et al* (2001) have reported a new Ft gene, the mitochondrial Ft (MtF). Mitochondrial Ft is a novel H-type Ft encoded by an intronless gene on chromosome 5q23.1. It exhibits more than 75% sequence identity to the H-Ft gene and appears to sequester iron more avidly than cytosolic H-Fts. Experiments with transfectant cells demonstrated that the protein is synthesised as a precursor of about 30 kDa and is targeted to mitochondria by a leader sequence of 60 amino acids where is processed into a typical Ft shells. Mitochondrial Ft does not appear to be an obligatory intermediate in transfer of iron to heme and other iron compounds in mitochondria. Although, iron does not normally accumulate in mitochondria, defects in its transport can result in mitochondrial iron-loading. Mitochondrial Ft levels show to increase dramatically when heme synthesis is inhibited in normal erythroblasts (Levi *et al*, 2001 and Corsi *et al*,

2002). In general, very little is yet known about how iron is delivered to mitochondria and whether iron is accessible to MtF. Nevertheless, a study, by Corsi *et al* (2002) showed that in HeLa cells, although the levels of MtF were not increased by exogenous iron, when increased by transfection, MtF appeared to retain a high proportion of available iron. So iron is potentially as accessible to MtF as it is to cytosolic Ft and therefore MtF may provide a new powerful method for regulating cellular iron homeostasis.

*1.3.4* New genes in iron metabolism

Exciting additions to our understanding of iron homeostasis have come with the recent identification of the hemochromatosis protein HFE, the transferring-independent transporter Nramp2, the iron exporter ferroprotin 1and hephaestin.

Hereditary hemochromatosis (HH) is a common autosomal-recessive disorder of iron metabolism with an inherited disorder that results from accumulation of excess iron in many organs. More than 80% of HH patients have a mutation in a major histocompatibility complex (MHC) class I type protein (HFE). The first indication of how HFE might regulate iron metabolism came with the discovery that it associates with the TfR (Waheed *et al*, 1999). HFE is a glycoprotein, which associates with microglobulin at the cell surface. The mRNA of HFE expression has an IRE and lack of HFE expression may be associated with iron overloading, but the exact mechanism by which HFE might facilitate iron homeostasis in the body still remains unknown. Recent indirect evidence by Riedel *et al* (1999) implied that HFE expression leads to activation of IRPs and results in decreased Ft and iron uptake from diferric Tf in HeLa cells.

A compelling candidate for endosomal iron transport has been recently identified by two groups, Flemming *et al* (1997) and Gunshin *et al* (1997). The transporter Nramp2 (natural resistance-associated macrophage protein) was first identified as a metal transporter-1 (DMT1) or divalent cation transporter-1 (DCT1) and is encoded by a gene belonging to the *Nramp* family of genes identified by Collier *et al* (1995). The mRNA of the protein has IREs at its 3'-UTR and is expressed in many different tissues with high expression levels at the duodenum brush border. The transporter Nramp2 is located on the plasma membrane as well as on subcellular compartments characterised as late endosomes or lysosomes. It is generally believed that Nramp2 is subcellulary localised with Tf and plays a role in transporting Tf-bound iron from the endosome (see secton 1.3.1 and review by Ponka *et al*, 2002).

Additional information on iron metabolism has also come with the cloning and characterisation of a gene referred to as ferroprotin-1/Ireg-1/MTP-1 (McKie *et al*, 2000). The localisation of ferroprotin-1 in cells and tissues is consistent with its proposed function of exporting iron from cells. The ferroprotin-1 mRNA possesses an IRE in the 5'-UTR. This stem loop structure is similar to other IREs found at the 5' end of mRNAs encoding Ft, 5-ALA synthase and mitochondrial aconitase, suggesting that ferroportin-1 could be down-regulated under conditions of low intracellular iron. Ferroportin-1 resides at the basolateral surface of duodenal enterocytes and may mediate iron efflux across membranes to plasma by a mechanism that requires ferroxidase activity (reviewed by Roy and Enns, 2000; Lieu *et al*, 2001).

Finally, sex-linked anaemia (sla) is a disorder that highlights the selectivity of the enterocyte basolateral iron transport machinery and emphasises its role in the regulation

of iron absorption. The protein product of the gene mutated in the sla mouse is the hephaestin, which has significant homology to the serum protein ceruloplasmin. Ceruloplasmin is a serum multicopper ferroxidase required for efficient iron recycling between storage and donation sites (Vulpe *et al*, 1999). Cloning of hephaestin led to the proposal of a role for copper in the transfer of iron from the enterocyte to the plasma.

## 1.4 Ultraviolet (UV) light

### *1.4.1* General remarks

At the turn of the $20^{th}$ century, although many photoreactions were known due to exposure of a substrate to (sun)light, the underlying principles were poorly understood. Light is a form of electromagnetic radiation that is characterized by its wavelength $\lambda$ (m) or its frequency v ($c^{-1}$), which are linked by the relationship: $v = c/\lambda$ , where c is the speed of light ($3x10^8$ $ms^{-1}$). The first law of photochemistry has been recognised by Grotthus (1817) and Draper (1843) who stated, "Only the light absorbed by a molecule can produce photochemical change in the molecule". But, it was not until Planck's work, in 1901, that a new relationship was described on the basis of light as being composed of packets of energy described as "light quanta" and as "photons" behaving as particles. So, the energy per photon (E) is determined by its wavelength as described by the relationship: $E = h \times v = h \times c/\lambda$, where h is Planck's constant ($6.626x10^{-34}$ Js). Consequently, Stark and Einstein modified the first law of photochemistry by stating that "absorption of radiation by species causes atoms or molecules to get excited through the electrons distribution to a higher energy arrangement". According to these principles, it

follows that, the shorter the wavelength, the higher is the energy of the photons of the electromagnetic radiation.

The sun continuously emits electromagnetic radiation over a broad range of energies, extending from wavelengths shorter than the UV through infrared and radio wavelengths. It is primarily the sun's surface temperature that determines the spectrum of energy it radiates. The spectral distribution of solar radiation that reaches the earth's surface ranges between 280 and 400 nm and is subdivided into the UVA (320–400 nm) and UVB (290-320 nm) as shown in **Figure 1.5**. UVA also called "no near-UV" comprises the major part (96%) of the total energy of solar UV radiation (Wilkinson, 1983). However, UVB also called "mid-UV" accounts for only 4% of solar UV. The third UV component of sunlight is UVC (190-290 nm) that does not reach the earth's surface since it is filtered by the stratospheric ozone layer and hence is of negligible intensity. Therefore the effects of solar UV radiation on biological systems are only considered in terms of UVA and UVB.

The UVB component is directly absorbed by cellular macromolecules including DNA and protein leading to DNA photodamage and mutagenesis. In contrast, UVA is weakly absorbed by most biomolecules but is oxidative in nature, generating ROS via interaction with intracellular chromophores (see section 1.4.3). In this thesis, only UVA that is the oxidizing component of sunlight will be discussed.

*1.4.2* Beneficial effects of UV radiation

The sun's rays provide warmth and light that enhance the general feeling of well-being and stimulate blood circulation. UV radiation is essential to the body as it stimulates the production of vitamin D. Vitamin D is essential for maintaining blood calcium levels within a normal and healthy range. Vitamin D is obtained either from the diet or by UV radiation of the skin (reviewed by Tyrrell, 1994).

UV radiation has been also used to successfully treat a number of diseases, including rickets, psoriasis, eczema and jaundice. Although, this therapeutic use cannot eliminate the negative side effects of UV radiation, treatment takes place only when its benefits outweigh the risks.

*1.4.3* Ultraviolet A-induced oxidative stress

Ultraviolet (UV) radiation must be absorbed in order to produce a chemical change. The first step in a photochemical reaction is the absorption of a single photon by a molecule, the chromophore and the production of an excited state in which one electron of the absorbing molecule is raised to a higher energy level. Several cellular components such as quinones, flavins, steroids and porphyrins are important UVA chromophores. The amino acids tyrosine and tryptophan, as well as NADH and NADPH, also exhibit absorption within the UVA range.

Evidence from *in vitro* studies have shown that UVA irradiation is a generator of intracellular oxidative stress (Danpure and Tyrrell, 1976; Tyrrell and Pidoux, 1989; Tyrrell *et al*, 1991; Vile and Tyrrell, 1995). These studies demonstrated that singlet

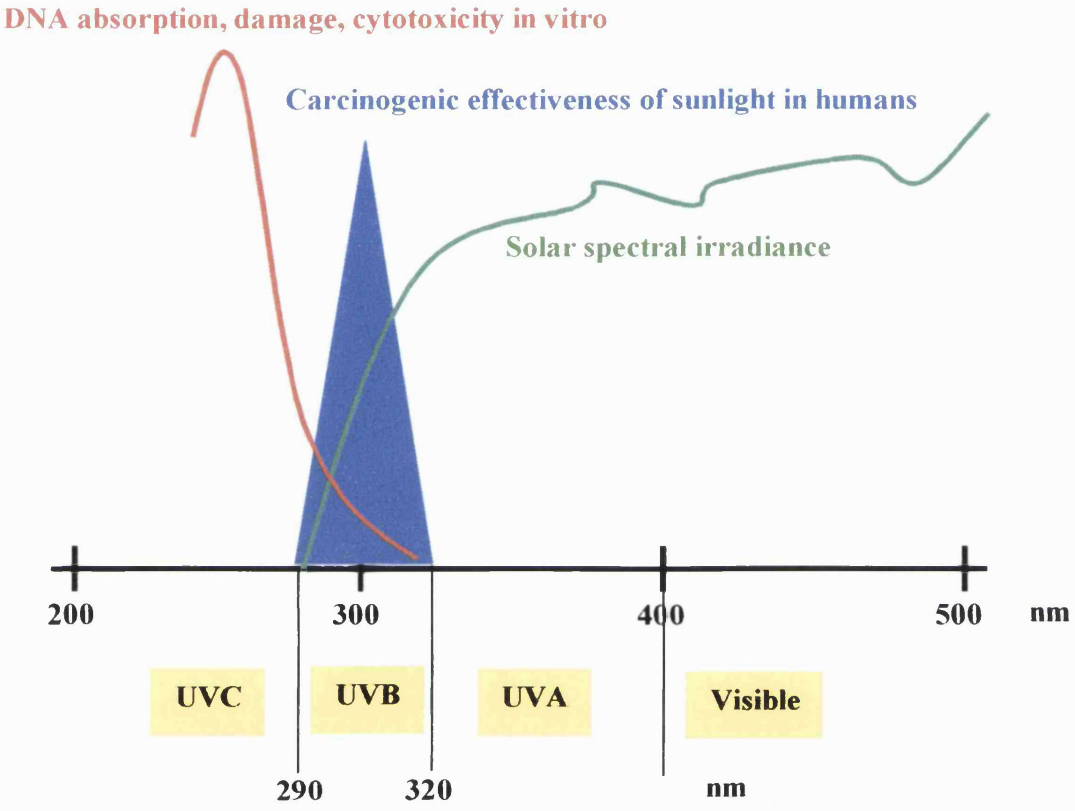



***Figure 1.5*:** Diagrammatic representation of the ranges of UV and visible radiation on the surface of the earth (Adapted from Tyrrell, 1994).

oxygen ($^1O_2$) and $H_2O_2$ are the most important ROS produced by UVA promoting biological damage in exposed tissues. The pathways by which several oxygen species may be generated by UVA are shown in **Figure 1.6**. The most important photolytic degradation product of tryptophan is $H_2O_2$ and $O_2^{\bullet -}$. Additionally, iron-catalysed reduction of $H_2O_2$ via either the Haber-Weiss or Fenton reaction can further generate the highly reactive $OH^{\bullet}$. The latter has also been supported by the *in vivo* studies of Badwey and Karnovsky in 1980 on relevant reactions generating $^1O_2$. The generation of $^1O_2$ after UVA treatment has also been supported by studies in human fibroblast cell lines using either deuterium oxide ($D_2O$, enhancer of $^1O_2$ lifetime) or sodium azide ($^1O_2$ quencher) in the presence of UVA irradiation. These studies revealed that $D_2O$ sensitised cells to UVA cytotoxicity whereas sodium azide strongly protected the cells against the lethal action of UVA (Tyrrell and Pidoux, 1989). Another important pathway of generation of intracelular ROS by UVA radiation involves photo-oxidation of NADH and NADPH (see **Figure 1.6** and Cunningham *et al*, 1985). Finally, more recent studies from this laboratory using specific scavengers of $^1O_2$ have clearly demonstrated the involvement of $^1O_2$ in UVA cytotoxicity to mammalian cells (Tyrrell *et al*, 1991).

Anderson and Parrish in 1981 have confirmed that melanin (complex polymeric protein produced by melanocytes) is another important UVA absorbing chromophore in human skin. Human melanoma cells with high melanin content have been shown to accumulate twice as much oxidative damage upon UVA radiation than cells with low melanin content (Kvam and Tyrrell, 1997).

It is now well known that UVA is a strong membrane damaging agent. Several reports have demonstrated that UVA irradiation can peroxidize membrane lipids (Putvinsky *et* al,

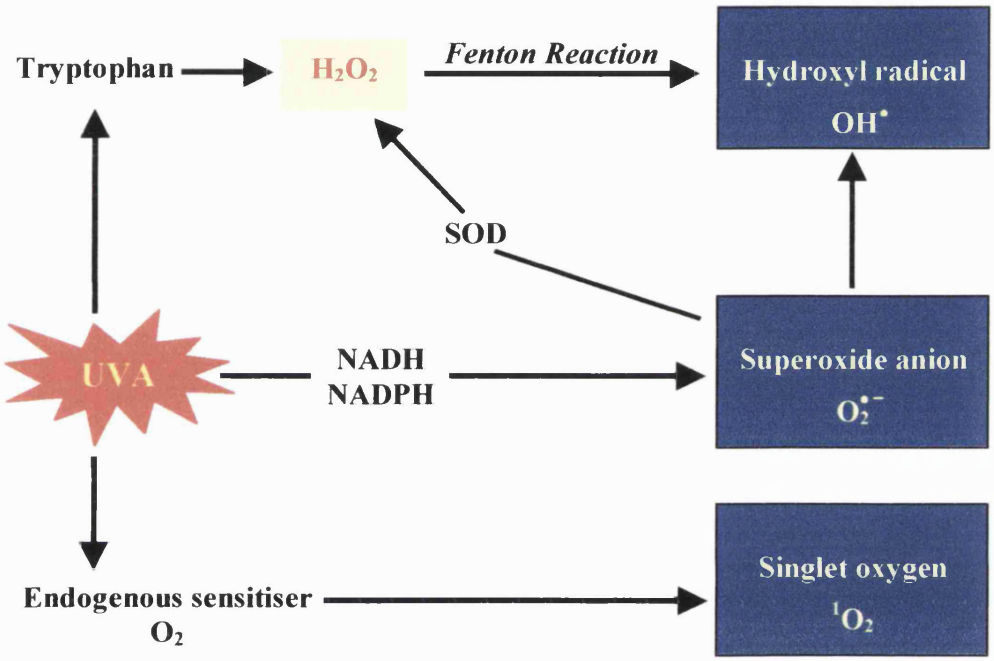



***Figure 1.6*:** Schematic presentation of the $O_2$ intermediates produced by UVA (Adapted from Tyrrell *et al*, 1991).

1979 and Azizova *et al*, 1980). Physiological doses of UVA can induce lipid peroxidation (production of alkoxyl radical (LO$^{\bullet}$), peroxyl radical (LOO$^{\bullet}$) and lipid peroxide (LOOH)) in membranes of human primary fibroblasts and keratinocytes via pathways involving iron and $^1O_2$ (Morliere *et al*, 1991, Punnomen *et al*, 1991 and Vile and Tyrrell, 1991).

UVA-induced lipid peroxidation was also found to be dependent on the chemical composition of membranes, as polyunsaturated fatty acid enrichment of human keratinocytes increased the peroxidation process (Quiec *et al*, 1995). The UVA-induced membrane damage has also been directly correlated with cell death in human skin fibroblasts (Applegate *et al*, 1994). Internal lipid membranes in eukaryotic cells such as those of lysosomes, mitochondria and nucleus have also been shown to be damaged following UVA radiation (see section 1.7).

Furthermore, other immediate effects of UVA irradiation include depletion of intracellular glutathione (GSH) and alteration in gene expression. Tyrrell and Pidoux in 1988, suggested that UVA radiation cytotoxicity is directly related to endogenous GSH levels. This was based on the observation that GSH depletion of human skin fibroblasts with buthionine-S,R-sulfoximine (BSO) could sensitise the cells to UVA radiation. Non-toxic concentrations of BSO reduced cellular GSH by inhibiting a critical enzyme in GSH synthesis, γ-glutamyl cysteine synthetase. These studies also provided evidence for the role of GSH in protecting cells against UVA damage possibly by acting as a H donor for glutathione peroxidase (GPx) and hence, reversing lipid peroxidation (see section 1.4.4). In 1992, Lautier *et al* have further demonstrated that depletion of intracellular GSH could also enhance the accumulation of heme oxygenase (HO) mRNA in human

skin fibroblasts after treatment with UVA radiation, supporting the hypothesis that cellular reducing equivalents are critical to UVA modulation of gene expression.

### 1.5 Heme oxygenase (HO)

Several studies have demonstrated that different forms of oxidative stress, including UVA radiation and $H_2O_2$, are capable of inducing gene expression in mammalian cells. Among these genes, HO has been shown to become highly activated under conditions of oxidative stress (Keyse and Tyrrell, 1989).

Heme oxygenase (HO) is a microsomal isozyme and one of its major functions is to catalyse the first and rate-limiting step in the oxidative degradation of heme to biliverdin, with the release of iron and carbon monoxide (CO) as shown in **Figure 1.7**. In turn, the biliverdin produced is converted to bilirubin in the cytosol. Bilirubin has been discovered as the most abundant endogenous antioxidant, since it accounts for the majority of the antioxidant activity of human serum (Gopinathan *et al*, 1994). In addition, HO is the major endogenous source of CO in the body. Carbon monoxide can act as a heme ligand and recent studies have demonstrated that it may have similar signalling functions to nitric oxide (NO) (Otterbein *et al*, 1999).

The active site of HO is located on the cytoplasmic site of the endoplasmic reticulum (ER) (Hino *et al*, 1979). In particular, there are three isoforms of mammalian HO: HO-1, an inducible enzyme that is most highly concentrated in tissues that are heavily involved in the catabolism of heme proteins; HO-2, a non-inducible isoform that is thought to be particularly involved in signalling pathways; and HO-3 which has low catalytic activity

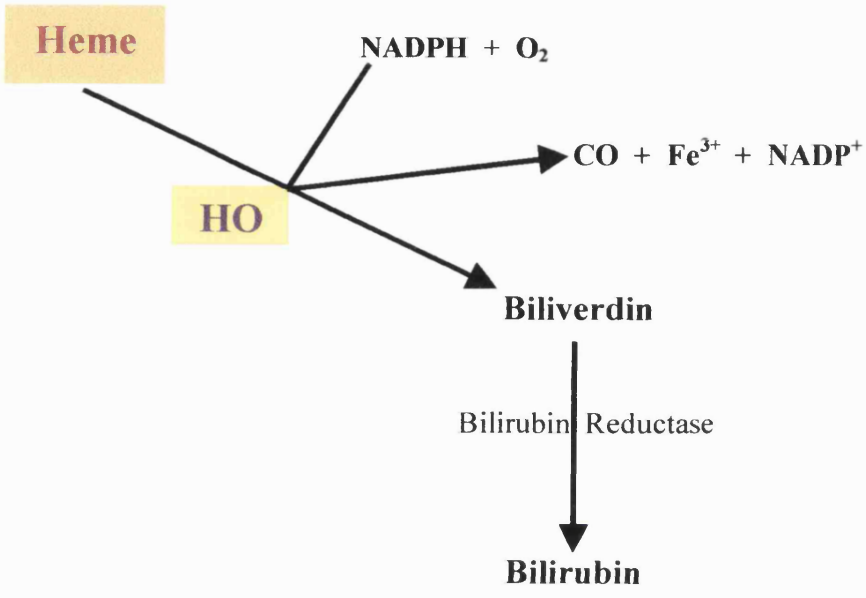



***Figure 1.7*:** Diagrammatic representation of heme degradation by HO.

and an uncertain physiological role (Maines *et al*, 1986; McCourbey *et al*, 1997; Ortiz de Montellano, P.R., 2000).

Although, heme is the typical HO inducer several studies demonstrated that HO enzyme activity could also be stimulated by a variety of stressful stimuli including oxidants such as UVA and $H_2O_2$. Cellular susceptibility to oxidant-mediated killing is greatly increased by heme since it can readily enter the cell membrane and catalyse the oxidation of low-density lipoprotein (LDL) (Balla *et al*, 1991; Miller and Shaklai, 1994; Camejo *et al*, 1998). Deficiency of the heme catabolysing enzyme, HO-1, has also been shown to result to an abundance of the circulating heme. Recent studies (Jeney *et al*, 2002) in human endothelial cells also indicated that when hemoglobin gets oxidised it can become directly cytotoxic through the generation of oxidised LDL due to the release of heme. As a result, both HO-1 and Ft are induced presumably as part of the intracellular antioxidant defence system.

Observations made in HO-1 deficient mouse and human have highlighted the important metabolic and cytoprotective role of this gene. Deletion of the mouse HO-1 gene resulted in incapacity of mice to modulate body iron stores properly and were less resistant to hepatic injury by iron, indicating that HO-1 plays an important role in iron utilisation (Poss and Tonegawa, 1997).

HO-1 has also been shown to play a critical role in cardio-protection as it can partially rescue the heart from ischemia/reperfusion injury by functioning both as an intracellular antioxidant and inducer of its own expression under stressful conditions (Yoshiba *et al*, 2001). Furthermore, HO-1 is clearly a redox-regulated gene, since not only it is induced by several oxidants (Keyse and Tyrrell, 1989), but also because both

basal and oxidant-induced levels of HO-1 accumulation are strongly increased by depleting cellular GSH (Lautier *et al*, 1992). UVA mediated activation of the HO-1 gene in human skin fibroblasts was efficiently blocked by $^1O_2$ quenchers such as sodium azide and was enhanced when cells were irradiated in the presence of deuterium oxide ($D_2O$) (Basu-Modak and Tyrrell, 1993).

In addition to HO-1, the other two constitutive isoforms of heme oxygenase, HO-2 (36 kDa) and HO-3 (33 kDa) have also been studied although their exact function has yet to be elucidated. So far, studies by Rotenberg and Maines (1991) and McCourbey *et al* (1997, 1993) have revealed that the amino acid sequence between HO-1 and HO-2 is around 40% similar and both isoforms display the same enzymatic activity and hence the molecular mechanism of the enzyme action should be analogous. Ishikawa *et al* (1995) that expressed the human HO-2 protein in a bacterial expression system, suggested that the HO-2 catalytic mechanism of heme degradation is very similar to HO-1. Finally, in an HO-2 gene-deletion mouse model, HO-1 induction increased oxidative damage during hyperoxia by mechanisms that appeared to involve a two-fold increase in lung GSH and accumulation of redox active iron (Dennery *et al*, 1998), suggesting an indirect role for HO-2 in induction of oxidative damage.

The function of the third isoform of heme oxygenase (HO-3) still remains unknown. The only proposed mechanism regarding its function is that since it contains a heme regulatory motif, it might be a heme sensing/binding protein (reviewed by McCourbey *et al*, 1997).

## 1.6 Cellular defence mechanisms

Because of the involvement of ROS in damaging effects much attention has been drawn to the cellular antioxidant systems and their importance in prevention or removal of the damage. Cellular defence mechanisms include non-enzymatic and enzymatic systems, which sometimes act in synergy. A non-enzymatic defence molecule is GSH. Of the known enzymatic antioxidant systems, the best characterised are GPx, superoxide dismutases and catalase, which directly metabolise free radicals. Finally, the inducible protective pathways are also of essential contribution to cellular defence.

### *1.6.1* Non-enzymatic antioxidant defence

Glutathione (GSH), a ubiquitous tripeptide, is present in most mammalian cells at high concentrations and is believed to play a crucial role in the protection of cells against oxidative damage (Meister and Anderson, 1983). GSH biosynthesis is controlled by multiple enzyme systems. The first step of GSH biosynthesis is catalysed by γ-glutamyl cysteine synthetase (γ-GCS), a heterodimer consisting of a 73 kDa heavy subunit (γ-GCSh) and a 28 kDa light subunit. Although, the γ-GCSh subunit contains the entire catalytic activity, its activity can be modulated by the association with the light subunit, also called the regulatory subunit (Meister, 1988 and O'Brian and Tew, 1996). GSH is a powerful radical scavenger and acts as the unique hydrogen donor for protective enzymes such as GPx and GSH transferase (GST), resulting in the formation of GSH disulphide (GSSG or oxidised glutathione) (Tyrrell, 1994). GSSG is reduced to GSH by glutathione reductase (GR) in the presence of NADPH as the hydrogen donor.

Previous observations by Tyrrell and Pidoux (1986 and 1988) in cultured human skin fibroblasts and keratinocytes, have shown an increase in sensitisation of cells to the lethal effects of UVA by inhibiting GSH metabolism. GSH was depleted by treatment with buthionine S-R Sulfoximine (BSO), which reduces the cellular GSH levels via inhibition of the critical enzyme in GSH synthesis, γ-GCS. Also, GSH has been shown, by Fischer-Neilsen *et al* (1992 and 1993), to protect Chinese Hamster Ovary (CHO) cells against UVA irradiation. Recent studies by Chang *et al* in 2002, also showed a close association between GSH and protection of T lymphocytes against oxidative damage. Significant increase in GSH levels in Jurkat T cells was found to decrease the formation of ROS and activated the induction of apoptosis.

In addition to GSH, other antioxidant vitamins, such as vitamins C and E, may play a protective role against UVA induced oxidative damage. Vitamin E comprises at least eight isomers of tocopherol and is a major lipophilic antioxidant. It acts during lipid peroxidation by donating labile hydrogen to terminate propagating $LO^{\bullet}$ and $LOO^{\bullet}$ groups and other radicals such as $O_2$ and OH . Vitamin C is a hydrophilic antioxidant and reacts directly with a wide range of ROS (Haliwell and Gutteridge, 1999). It is able to quench $^1O_2$ (Chou and Khan, 1983) and it can restore properties of oxidised vitamin E. Both vitamin E and vitamin C have complementary roles in preventing lipid peroxidation induced by oxidative stress, as reviewed by Tyrrell, 1994.

*1.6.2* Enzymatic defence-antioxidant enzymes

The major antioxidant enzymes include catalase which destroys $H_2O_2$, superoxide dismutase (SOD) which converts $O_2^{\bullet -}$ to $H_2O_2$ and GPx that metabolises $H_2O_2$ and reduces lipid peroxidation radicals such as LOOH.

Catalase is an enzyme that is located in the peroxisomes. Catalase destroys $H_2O_2$ by catalysing the direct decomposition of $H_2O_2$ to ground state oxygen and water. Also, there is evidence showing that catalase activity is strongly inhibited after UVA exposure, of cultured human fibroblasts and keratinocytes (Punnonen *et al*, 1991; Moysan *et al*, 1993; Tirache *et al*, 1995; Shindo *et al*, 1997). However, comparing catalase activity to GSH, catalase is less important for the protection of cells against oxidative damage, since it has been shown that when cells are deficient in catalase, there is no decrease in cell survival after UVA radiation (Tyrrell and Pidoux, 1989; Peak *et al*, 1990).

SODs are located in the cytosol, mitochondria and plasma membranes, and catalyse the reduction of $O_2^{\bullet -}$ to less reactive $H_2O_2$. The activity of SOD varies among the tissues and its activity is regulated through biosynthesis, which is sensitive to tissue oxygenation (Yu, 1994). Since SOD reduces $O_2^{\bullet -}$ to $H_2O_2$, the increase in SOD activity has been shown to be accompanied with an increase in catalase and/or GPx to prevent $H_2O_2$ toxicity (Amstad *et al*, 1991 and Yohn *et al*, 1991).

GPx is a selenium dependent enzyme, located in the cytosol and mitochondrial matrix. GPx acts by coupling the reduction of $H_2O_2$ and lipid peroxides with the oxidation of GSH, forming water and alcohol, respectively.

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

or $$2GSH + LOOH \xrightarrow{GPx} GSSG + H_2O + LOH$$

GPx according to studies by Lecia *et al* (1993) can also significantly decrease the level of UVA-induced oxidative membrane damage. Recently it has been shown that low doses of UVA radiation lead to an up-regulation of GPx activity, protecting cells against subsequent challenge of higher doses of UVA (Meewes *et al*, 2001).

*1.6.3* Inducible cellular defence

*1.6.3a* The protective role of ferritin

The catalytic role of iron in the generation of ROS anticipates the important role of Ft in modulating cellular sensitivity to oxidative stress. Early studies, by Balla and co-workers in 1992, revealed that heme induced Ft synthesis in endothelial cells and subsequently reduced their cytotoxic response to $H_2O_2$. During the following year, in tumour cell lines, it was demonstrated that sensitivity of these cells to oxidants was inversely correlated with Ft protein levels, since alteration in Ft levels with hemin could alter oxidant sensitivity (Cermak *et al*, 1993). In 1995, Cairo *et al* demonstrated that under conditions (*in vivo*) of oxidative stress, liver Ft can represent either a pro- or and anti-oxidant mode. Observations of the cooperative work between the cleavage by HO and Ft degradation to increase the intracellular LIP levels were also proposed. Indeed, expansion of LIP levels induced Ft at the transcriptional level and decreased IRP binding activity. There are also recent observations showing that increased Ft levels reduce the

levels of LIP (Picard *et al*, 1998). These studies were also supported by data showing that reduction in Ft sensitised cells to oxidant cytotoxicity. In brief, when cells overexpressed Ft, both the oxidant toxicity in the cells was reduced as well as the importance of H-Ft ferroxidase activity (Epsztejn *et al*, 1999 and Cozzi *et al*, 2000). Other prooxidant conditions including treatment with $H_2O_2$ also suggested the cytoprotective role of Ft, whose role in sequestering LIP limited oxidative damage induced by $H_2O_2$ (Cairo *et al*, 1995 and Cermak *et al*, 1993). Both transcriptional and post-transcriptional mechanisms have been implicated in Ft induction by oxidants. Transcriptional induction of H-Ft genes in rat livers and GSH reduction resulted in cellular defence against cytotoxicity of ROS formation (Cairo *et al*, 1995). Furthermore, studies by Lin and Girotti (1997) showed that hemin-stimulated cells had increased up to 3-times the levels of Ft molecule compared to nonstimulated controls. These findings supported the hypothesis that induced Ft, enriched in H-chain, sequesters redox active iron and thereby enhances resistance of cells to oxidants. Finally, studies on the contribution of IRP1 inactivation through oxidation of critical cysteine residues leading to Ft induction were very contradictory on the protective role of Ft against oxidative stress (Pantopoulos and Hentze, 1995 and 1998).

*1.6.3b* The role of heme oxygenase

The regulation of HO is an excellent model of a cellular pathway that has evolved to protect cells against oxidative stress. The inducible form of HO, the HO-1, has been shown to play a crucial role in protection of the cells against the toxic effects of different oxidising agents such as UVA and $H_2O_2$ (Keyse and Tyrrell, 1987 and 1989, Lautier *et al*, 1992 and Vile and Tyrrell 1993). Moreover, recent studies by Ferris *et al* (1999) also

showed that absence of HO-1 leads to iron accumulation, whereas HO-1 overexpression decreases cellular iron levels. Protection of cells by HO-1 parallels a decrease in intracellular iron levels, and HO-1's protection of cells following oxidative stress is mimicked by iron chelation.

Interestingly, it is now well recognized that the enhanced protective role of HO-1 is central to the development of an adaptive response that involves Ft. The overall effect of HO is to remove the pro-oxidant heme while generating the anti-oxidant, bilirubin, and another pro-oxidant, iron, that will be taken up by Ft. Vile *et al* in 1994 clearly demonstrated that when human skin fibroblasts were treated with HO-1 anti-sense oligonunleotides, the UVA-induced increase in Ft levels was prevented as well as the adaptive response that leads to protection against oxidative damage. An additional study by Rothfuss *et al* (2001) in human lymphocytes also demonstrated the functional involvement of HO-1 against the induction of oxidative DNA damage, but the exact mechanism remains to be elucidated.

### 1.7 Concluding remarks on the role of iron in oxidative stress

Pourzand *et al* (1999) have provided the first evidence that UVA radiation leads to an immediate increase in the "free" iron mainly as a result of degradation of Ft molecule. Indeed, the immediate decrease in IRP1 binding activity after UVA radiation coincided with a significant decrease in Ft levels. The lack of Ft would be expected to further exacerbate the damaging effects of LIP release in human cells. Pourzand *et al* (1999) also showed that Ft degradation that occurs immediately after UVA radiation originates from destabilisation of lysosomal membranes and the subsequent leakage of proteolytic

enzymes from these organelles. This results in the release of "free" iron in the cells, which is potentially harmful (**Figure 1.8**).

Iron is also liberated from Ft as a consequence of normal turnover in lysosomes, where it is recycled for heme synthesis. According to Vaisman *et al* (1997) intracellular Ft donates iron for heme synthesis. Ferritin degradation caused by lysosomal damage is necessary for iron release and its transfer to heme. In addition to UVA-induced increase in LIP, Kvam *et al* (1999) have also demonstrated that UVA radiation induces the release of free heme within microsomal membranes of skin fibroblasts, resulting in the sensitisation of cells to a further exposure to UVA. Heme itself is not a source of the LIP, but it is a substrate for heme-catabolising enzyme, HO, which could release the heme iron.

Support for the concept that the induction of HO is an immediate response to break down heme has been provided by further studies with human skin fibroblasts. In particular, Noel and Tyrrell (1997) demonstrated that there is a refractory response to UVA activation of HO-1 expression that gradually develops to a maximum at 48h following the initial irradiation. This led to the conclusion that long-term activation of HO-1 expression can be damaging to the cell and therefore a mechanism for down-regulation is required.

Previous studies from this laboratory (Keyse and Tyrrell, 1989; Vile and Tyrrell, 1993; Vile *et al*, 1994) have demonstrated that UVA radiation induces strong transcriptional activation of HO-1 gene in human primary skin fibrobasts, which eventually modulates Ft levels as a way to protect cells against UVA-induced oxidative damage.

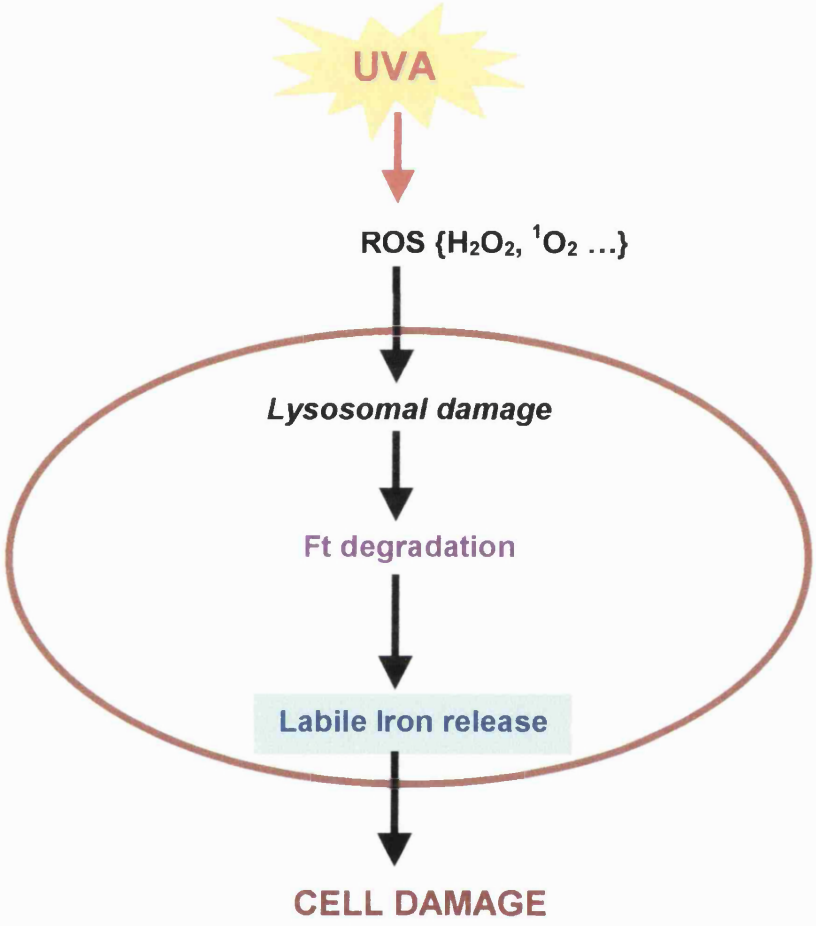



***Figure 1.8***: Diagrammatic representation of the main steps involved in the UVA-mediated damage in human skin cells, as proposed by Pourzand *et al* (1999).

In summary, cytoprotective effects of HO may be due to several functions: first, because increased levels of heme enhance the prooxidant state of the cell, the degradation of heme by HO may be an adaptive mechanism in response to oxidative stress. Secondly, HO may exert its antioxidant function via the production of bilirubin, which is a very efficient scavenger of free radicals. And finally, HO-1 mediated induction of Ft has been accepted as an additional adaptive response of cells to oxidative stress as intracellular free iron is sequestered by Ft and the induction of Ft by oxidants appears to be coupled to induction of HO-1 (Fogg, *et al*, 1999).

In addition to UVA, $H_2O_2$ is another form of oxidizing agent. It is well known that when cells are exposed to external $H_2O_2$, the $H_2O_2$ rapidly diffuses inside the cell and oxidises $Fe^{2+}$, thereby forming $OH^{\bullet}$. Once inside the cells $H_2O_2$ can degrade certain hemoproteins, including myoglobin, hemoglobin and cytochrome c, promoting the release of iron inside the cells. For example, a study by Stridh *et al* (1998) demonstrated that there is a cytochrome c release and caspase activation in $H_2O_2$ treated cells that result in apoptosis (see section 1.8). Following this observation, Antunes *et al* (2001) and Yu *et al* (2003) suggested that cytochrome c release is probably due to the enzymes activated by lysosomal rupture. These data showed that a low steady-state concentration of $H_2O_2$ can cause lysososmal rupture, which after several hours leads to the induction of apoptosis. Their hypothesis was also supported by pre-treatment of cells with the iron chelator desferrioxamine (DFO), which inhibited both lysosomal rupture and apoptosis and correlated the involvement of intralysosomal iron as a major determinant of $H_2O_2$ induced cell death.

The reactivity of $H_2O_2$ with iron intimately connects oxidative stress and cellular iron metabolism. Studies from Pantopoulos and Hentze (1995) and Pantopoulos *et al* (1997), indicated that exposure of cells to $H_2O_2$ leads to reduced synthesis of Ft and stimulates TfR mRNA expression, which mediates iron uptake into the cells. Both responses were triggered via the induction of IRP1 binding activity following $H_2O_2$ treatment. The regulation of Ft and TfR expression by $H_2O_2$ via IRP1 provided a novel pathway in response to oxidative stress in mammalian cells. However, the data also suggested that IRP1 responds differently to oxidative stress when the oxidizing agent ($H_2O_2$) is applied exogenously as compared to endogenous generation of $H_2O_2$ i.e exogenous $H_2O_2$ treatment resulted in rapid activation of IRP1, while elevation of intracellular $H_2O_2$ levels were not sufficient for the induction of IRP1.

In general these findings demonstrate that the effects of $H_2O_2$ on cellular iron metabolism are complex and involve both IRP1-dependent and independent mechanisms that need to be investigated further.

Recent work from our laboratory have also showed that exogenous $H_2O_2$ promotes the release of "free" iron in the cytosol of human skin fibroblasts, although the exact pathway has not yet been identified (unpublished data).

*In vivo* work on the role of Ft ferroxidase activity (Cozzi, *et al*, 2000) demonstrated that the overexpression of H-Ft induces both iron deprivation and increased resistance to oxidative damage by $H_2O_2$.

Overall, oxidative stress, either in the form of UVA or in the form of $H_2O_2$, affects iron metabolism via an increase in LIP levels, a decrease in Ft levels and the release of free heme within microsomal membranes.

Finally, several studies have shown that UVA can cause damage to various cellular compartments and that endogenous antioxidant mechanisms influence most of the action of UVA radiation.

### 1.8 Consequences of oxidative stress: cell death

Cell's exposure to oxidative stress can lead to cell death. Cell death can occur essentially by two mechanisms, necrosis and apoptosis. Apoptosis and necrosis are two forms of cell death with clearly distinguishing morphological and biochemical features (Wyllie *et al*, 1980). Necrosis is the pathological process, which occurs when cells are exposed to a serious of physical or chemical insult whereas apoptosis or programmed cell death is the physiological process responsible for the elimination of superfluous, aged or damaged cells. Although apoptosis is necessary for both health and disease, necrosis is always the outcome of severe and acute injury.

Apoptosis and necrosis have long been considered as two distinct mechanisms of cell death, each with different biochemical and morphological characteristics (see **Table 1.1** and Wyllie *et al*, 1980; Majno and Jorris, 1995). Indeed, apoptotic cells are defined by fragmented nuclei with condensed chromatin, fragmented or condensed cytoplasm and formation of apoptotic bodies, whereas necrotic cells are characterised by electron-lucent cytoplasm, mitochondrial swelling and loss of plasma integrity without severe nuclear damage.

However, it is now widely accepted that apoptosis may also be triggered in response to toxic stimuli and oxidative stress (Dypbukt *et al*, 1994) or in association with necrosis (Staunton and Gaffney, 1998). In particular, it has been shown that it is the magnitude of

| Features | Apoptosis | Necrosis |
|---|---|---|
| **Morphological** | 1. Plasma membrane near-to-intact until late<br>2. Shrinking of cytoplasm and nuclear condensation<br>3. Fragmentation of cell into smaller bodies and formation of apoptotic bodies<br>4. Mitochondrial leakage | 1. Loss of membrane integrity<br>2. Early swelling of cytoplasm and mitochondria<br>3. Leakage of cell, complete lysis<br>4. Disintegration of organelles |
| **Biochemical** | 1. Energy (ATP)-dependent process<br>2. Release of various factors into cytoplasm by mitochondria<br>3. Activation of caspase cascade<br>4. Alterations in membrane asymmetry by translocation of phosphatidylserine from the cytoplasm to the extracellular | 1. No energy requirement process<br>2. Loss of regulation of ion homeostasis<br>3. DNA fragmentation |

***Table 1.1***: A comparison of different features of apoptosis versus necrosis.

the initial insult, rather than the type of the stimulus that plays a critical role in the decision of the cell to undergo either apoptosis or necrosis, proposing that apoptosis and necrosis may not necessarily be two independent pathways but may share common events, at least in early phases of cell death process and in the signal transducing pathway (Bonfoco *et al*, 1995; Shimizu *et al*, 1996; Hampton and Orrenius, 1997).

On the basis of these findings, it has now been recognised that the downstream controller capable of directing the cells toward either type of cell death is intracellular ATP (Ellerby *et al*, 1997) as well as caspase activation. In particular maintenance of high ATP favours apoptosis over necrosis whereas ATP depletion and inhibition of caspase activation transforms apoptosis into necrosis.

The first step in the induction of cellular death by various stimuli is the increase in mitochondrial membrane permeability. A dynamic megachannel multiprotein complex formed in the contact site between the inner and the outer mitochondrial membranes is the mitochondrial permeability transition pore (PTP). The PTP complex can function as a sensor of oxidative stress since PTP opening can be triggered by several effectors such as ROS, pH changes, reduced concentrations of adenine nucleotides (ATP, ADP) and collapse of mitochonrial membrane potential ($\Delta\Psi m$). As a consequence of mitochondrial dysfunction, soluble proteins contained in the mitochondrial intermembrane space (cytochrome c, apoptosis-inducing factor) are released through the outer membrane into the cytosol and activate a class of proteases, the caspases (cysteine aspartate-specific proteases) (reviewed by Kroemer *et al*, 1998).

In brief, once released cytochrome c initiates formation of the apoptosome, which is a complex of Apaf-1 (apoptotic protease activating factor-1) and caspase 9. Indeed

cytochrome c acts in concert with ATP to induce conformational change in Apaf-1 that allows it to bind to pro-caspase 9 and form the apoptosome. The apoptosome will act as an initiator of the caspases which will lead to the cleavage of cellular proteins and apoptosis. Activation of caspases is regulated directly or indirectly by members of the Bcl-2 family that appear to regulate the release of cytochrome c from mitochondria and the subsequent activation of the apoptosome.

The Bcl-2 family of proteins possesses both pro-apoptotic (Bax, Bad, Bid, Bak, Bcl-$X_S$, Bik, Bim, Hrk) and anti-apoptotic (Bcl-2, Bcl-$X_L$, Bcl-W, Mc1-1, A1) molecules. It is the ratio of anti- to pro- apoptotic molecules (i.e. Bcl-2/Bax) that determines the response to a death signal. Overexpression of Bcl-2 in cells has been reported to block cell death, the reduction of $\Delta\Psi m$ and the release of cytochrome c from mitochondria whereas Bax induces these changes. Reed and co-workers (1998) have suggested that Bax plays a crucial role in the opening of the PTP and dissipation of $\Delta\Psi m$ via the participation of either in the formation or regulation of the megachannel, whereas Bcl-2 blocks its process.

Bcl-2 is localised in the mitochondrial membrane and therefore accumulation in its function can be associated with oxidative membrane damage. As already mentioned in previous sections, UVA is a strong membrane damaging agent and induces peroxidation of membrane lipids in human skin cells. Pourzand *et al* (1997) demonstrated that overexpression of Bcl-2 inhibits UVA-mediated immediate apoptosis, supporting the notion that Bcl-2 apart from its antiapoptotic function, possesses antioxidant properties. They also showed that expression of Bcl-2 dramatically decreases the level of HO-1 induction following UVA radiation.

The same spectrum of protection of Bcl-2 against oxidative damage has also been supported by the overexpression of Bcl-$X_L$ in astrocytes. In this study, they demonstrated that overexpression of Bcl-$X_L$ protects astrocytes from oxidative damage and is associated with higher GSH, Ft and iron levels (Xu *et al*, 1999).

Singlet oxygen and superoxide anion are two generators of UVA that have been shown to activate the PTP, which in turn triggers immediate apoptosis (Godar, 1999). The author proposed a model in which opening of the PTP sites by $^1O_2$ and superoxide anion provokes the release of AIF and cytochrome c release from mitochondria. In addition, a study in human HL-60 leukemic cells showed that an early decrease in $\Delta\Psi m$ following UVA radiation, activated caspase 3 and subsequently apoptosis (Tada-OiKawa *et al*, 1998).

Although most current studies on oxidative stress-induced apoptosis focus on caspases, mitochondrial energy changes and plasma membrane-bound death receptors, evidence has been brought forward for the role of lysosomes in the initiating phase of apoptosis (Brunk *et al*, 1997; Brunk and Svensson, 1999). Recent studies by Zhao *et al* (2003) have demonstrated that exposure of human skin fibroblasts to oxidant stress caused early release of lysosomal enzymes promoting the release of ctytochrome c due to enhanced mitochondrial oxidant production and initiation of the apoptosis.

Moreover, a study by Antunes *et al* (2001) demonstrated that apoptosis as a consequence of lysosomal rupture is also induced by a low steady-state concentration of $H_2O_2$. These data showed that a short exposure (15 min) to $H_2O_2$ caused the immediate lysosomal damage and later apoptosis and this could be inhibited by the use of desferioxamine (DFO), an iron chelator which is known to be taken up by endocytosis

and become localised in the lysosomes. These findings also proposed a link between intr-lysosmal iron-catalysed oxidative damage and induction of apoptosis.

However, it is important to mention that recent study presented by Brunk in the European Iron Meeting (Brunk, T., Yu, Z., Persson, L. and Eaton, J.W., (2003), Lysosomes, Iron and Oxidative stress, European Iron Club, Vienna) showed that when Jurkat T cells were exposed to higher doses of $H_2O_2$ and for a prolonged time, no induction of apoptosis could be detected. In support of this study data from our laboratory have also clearly demonstrated that UVA and $H_2O_2$ treatment of human skin fibroblasts causes a dose dependent induction of necrosis rather than apoptosis (unpublished data, this laboratory). Study of this thesis will also focus on the identification of the mode of cell death induced by two different forms of oxidative stress, UVA and $H_2O_2$ (see chapters 3 and 4).

### 1.9 Multidrug resistance (MDR)

In cancer treatment, one of the major problems to be overcome is the resistance of tumour cells to anticancer drugs. An intensively studied type of cellular drug resistance is the multidrug resistance (MDR) phenotype. Multidrug resistance is the phenomenon where cells exposed to one drug often develop resistance not only to that drug, but also to other drugs to which they have never been exposed.

*In vitro* studies have shown that MDR is accompanied by reduced intracellular drug accumulation due to increased efflux via energy dependent trans-membrane drug transport proteins (Endicott and Ling, 1989). These trans-membrane transport proteins

belong to the ABC transporter superfamily because each member contains a highly conserved ATP-binding cassette (ABC).

Over 50 ABC transporters have been described, and although most of the members have been described in prokaryotes, an increasing number are being discovered in eukaryotes. The first of these to be identified were discovered because of their ability to pump hydrophobic drugs out of eukaryotic cells. Members of the ABC family include the P-glycoprotein (PgP), the multidrug resistance protein (MRP) and a newly described mitoxantrone resistance protein (MXR or ABCG2). Another protein is the lung resistance protein (LRP), which although is not an ABC transporter, it is frequently involved in discussions of drug resistance. These act as ATP-dependent pumps, pumping anti-cancer drugs out of the cells and thus decreasing their intracellular accumulation and therefore their effectiveness to treat cancer cells.

### *1.9.1* The P-glycoprotein (PgP)

The multidrug transporter, PgP is a transmembrane 170 kDa glycoprotein, encoded by the mdr1 gene and overexpressed in various drug-resistant tumour cells. PgP is comprised of two similar halves (43% sequence homology), each containing 6 transmembrane domains, three glycosylation sites on the first extracytoplasmic domain and an ATP-binding consensus motif (Chen *et al*, 1986). PgP functions as an energy dependent efflux pump responsible for the removal of lipophillic and natural compounds out of tumour cells (Endicott and Ling, 1989).

P-glycoprotein was initially isolated due its role in MDR to cancer chemotherapeutics. In recent work, however, it is evident that this transporter is also

involved in the pharmacokinetics of many drugs. P-glycoprotein is expressed in many cells and is often associated with drug absorption and disposition.

Several mechanisms have been suggested to explain the PgP transport function. The model of Higgins and Gottesman (1992) proposed that PgP encounters xenobiotics in the inner leaflet of the plasma membrane and flips the agents to the outer leaflet, where they diffuse into extracellular region (**Figure 1.9**) and therefore promoting the translocation of a wide variety of short-chain lipid molecules (Helvoort *et al*, 1996). P-glycoprotein has also been postulated to increase intracellular pH via depolarising plasma membrane electric potential of the cell by acting as a proton pump, or a chloride channel, thus reducing intracellular accumulation of weak bases or reducing pH-dependent binding of agents to their intracellular targets (Roepe *et al*, 1993). Hence, PgP appears to be itself capable of direct substrate transport.

P-glycoprotein expression has been studied in many tumours, since its overexpression in tumour cells results in reduced intracellular accumulation of anticancer drugs including arthracyclines and vinca alkaloids and enhanced drug efflux and thus contributes to MDR. Treatment of an MDR human hepatoma HepG2 cell line with antisense RNA against mdr1 gene resulted in inhibition of PgP expression and reduction in drug resistance (Chan *et al*, 2000).

Much interest is focused on identifying chemical agents that can antagonise drug transport by PgP. To this regard, Mardeuf-Gueye *et al* (2000) have demonstrated a new compound (PAK-104P) that can inhibit the drug efflux by two mechanisms: a first mechanism involving a low affinity site for PAK-104P and which efflux does not depend

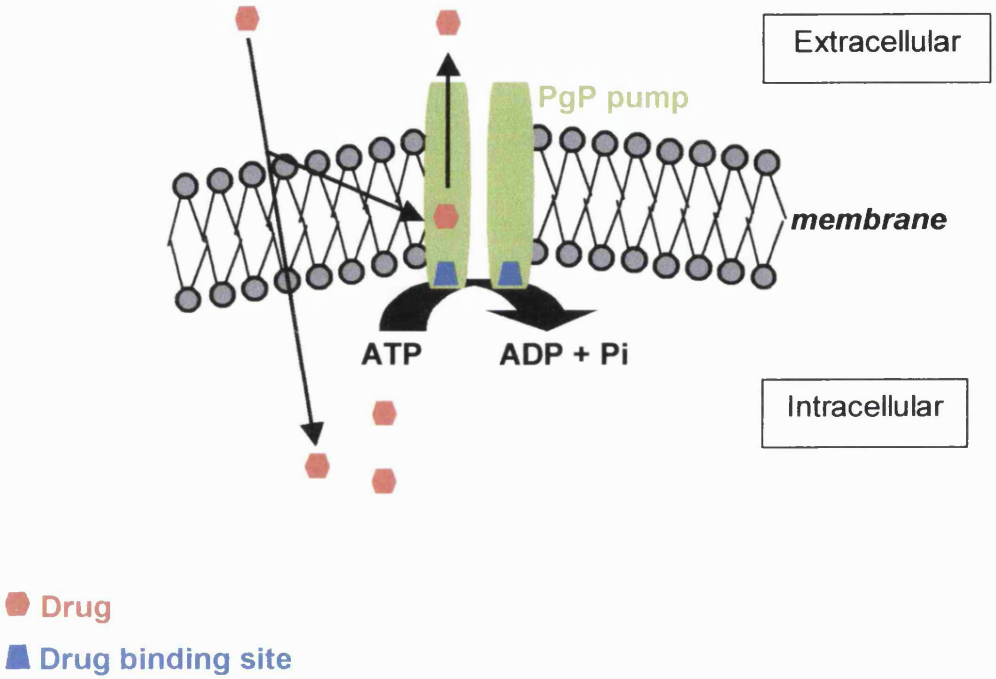



***Figure 1.9***: Diagrammatic representation of the proposed model by Higgins and Gottesman (1992) on the function of the PgP pump. (Adapted from Krishna and Mayer, 2000)

on GSH. A second mechanism involving a high affinity site for PAK-104P, which efflux depends on the presence of GSH.

Wigler and Patterson (1993) have demonstrated that inhibitors of PgP can resensitise MDR cells and hence PgP inhibitors have been used as modulators of MDR. The PgP reversing agent, verapamil, can modulate resistance in MRP overexpressing cells (Lautier *et al*, 1996). In addition, studies of a PgP negative multidrug resistant cell line led to the identification of another MRP.

The function of PgP as a transporter of known substrates is utilised in functional assays of PgP detection. The earliest assays included radiolabelling of anticancer drugs such as vincristine and doxorubicin but lately new methods were developed based on fluorochromes as PgP substrates (i.e. rhodamine 123 and 3,3'-diethyloxacarbocyanine iodide $DiOC_2(3)$) using flow cytometry. However, diversities in fluorescence can not always be linked to different levels of PgP expression, since other transport mechanisms may contribute to the drug or dye efflux. Therefore, combined incubation in the presence or absence of selective inhibitors of PgP function provides increasing specificity of the functional Pgp assays (Feller *et al*, 1995).

There are various agents that antagonise MDR by increasing the drug accumulation in MDR cells, whereas they show little or no effect on drug-sensitive cells. Most of these reversing agents, such as verapamil and cyclosporine are substrates for PgP mediated transport and competitively inhibit PgP transport of anti-tumour drugs.

P-glycoprotein is also present in various normal tissues such as gastrointestinal tract, liver, kidney as well as brain. P-glycoprotetin activity in normal tissues suggests an important role in transepithelial transport. Recent studies have shown that PgP is also

expressed in keratinocytes and might play a role in regulating the level of environmental toxins and differentiation factors (Sleeman *et al*, 2000).

*1.9.2* The multidrug resistance protein (MRP)

The MRP is a 190 kDa transmembranous glycoprotein which was identified by Cole *et al* (1992). MRP has been mainly identified in drug resistance of haematological malignancies, lung cancers, acute lymphoblastic leukaemia relapses and chronic myeloid leukaemia, but its contribution to chemoresistance of other tumours such as melanoma are under investigation.

Although MRP is similar to PgP in terms of substrate specificity, only 15% amino acid homology exists between the two proteins. Functionally, MRP differs from PgP in being capable of transporting organic anion drug conjugates as well as intact anticancer drugs (Cole *et al*, 1992; Loe *et al*, 1996). Unlike PgP, it has not been possible to demonstrate that MRP binds and actively transports unmodified forms of the drugs to which it confers resistance. The ability of MRP to function as an ATP-dependent membrane transport of anionic conjugates of endogenous and exogenous substance from cells into the extracellular space at a sufficient rate has been characterised by many cell types (**Figure 1.10**) (Konig *et al*, 1999). Specifically, the transport and detoxification of substances (e.g drugs, carcinogens) entering the cells involve firstly the oxidation of the substance. Subsequently, biotransformation processes catalysed by GST conjugate the substance with GSH, resulting in the export of the conjugated substance by the MRP pump. Hence, MRP export mechanism mainly functions in synergy with the GSH/GST detoxification system.

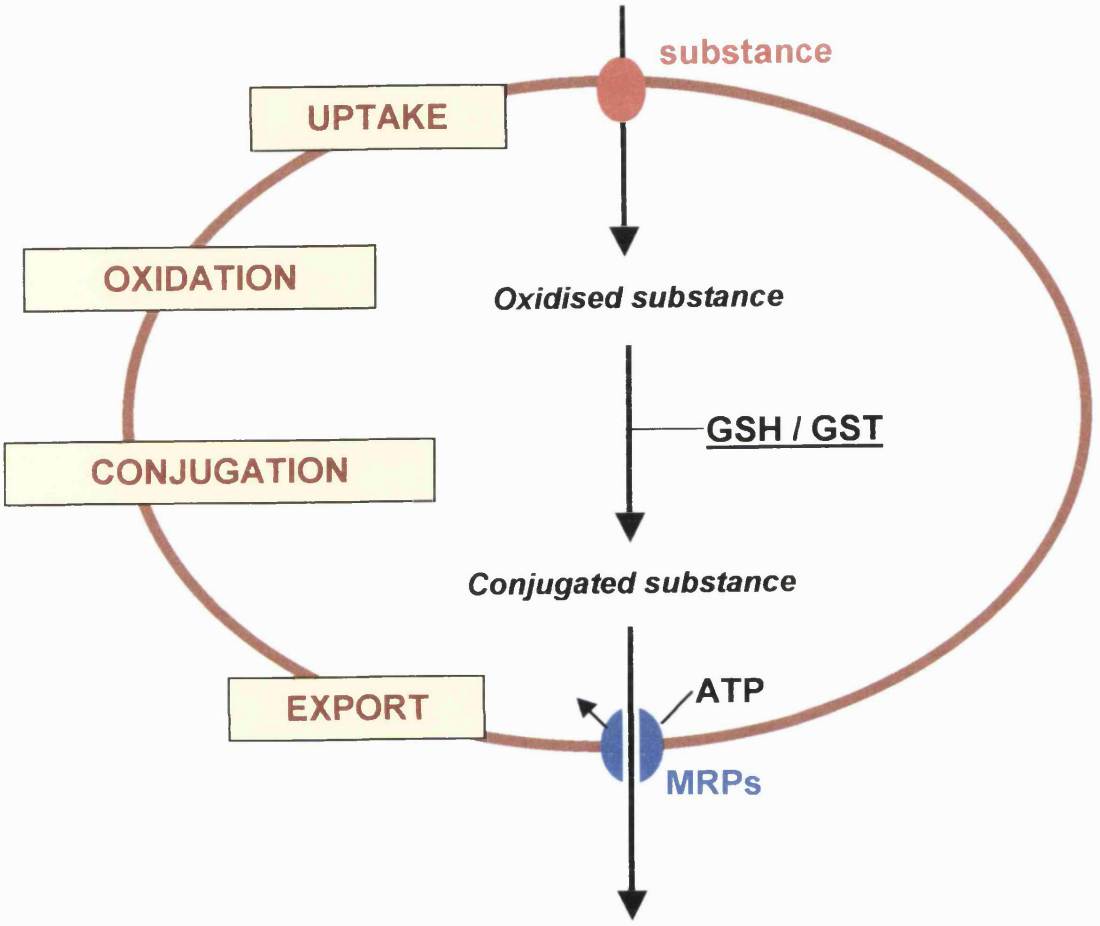



***Figure 1.10***: Diagrammatic representation of the MRP export mechanism. (Adapted from Konig *et al*, 1999)

Contribution of the MRP to the chemoresistance of melanoma cells is under investigation. In a recent study, Berger and coworkers (1997) have shown that in 50 cases of melanomas, resistance to daunomycin and doxorubocin, was entirely correlated with MRP gene expression.

MRP is also expressed in normal human tissues such as muscle, lung, spleen, bladder, adrenal gland and gall bladder (Zaman *et al*, 1993). Recently several isoforms of MRP have been identified, namely MRP1, MRP2 and MRP3-6. MRP1 and MRP2 function as organic anion transporters (Borst *et al*, 1997). MRP1 plays also a physiological role in the normal ATP-dependent membrane transport of GSH conjugates.

*1.9.3* The mitoxantrone (MXR or ABCG2)

The MXR has been recently identified by Miyake *et al* (1999) in a human colon cancer subline, S1-M1-80. It is a 655-amino-acid protein with six transmembrane domains and is encoded by the mitoxatrone resistance-associated gene mxr (Litman *et al*, 2000). Research on the examination of the phenotype of two mxr-overexpressing cell lines demonstrated a parallel phenotype with similar patterns of cross-resistance, matching drug distribution profiles and energy-dependent mitoxantrone efflux. This newly identified mechanism of resistance extends to numerous other anticancer agents and appears to be as potent as PgP in conferring MDR (Litman *et al*, 2000).

The MXR is an ABC half-transporter that is thought to dimerize to function and is expressed in high levels in the placenta. It has been shown to confer resistance to mitoxantrone and anthracyclines (Robey *et al*, 2001). In addition, MXR was also shown to play a role in the resistance of human breast cancer cells to flavopiridol, an inhibitor of

the MRP-mediated transport (Robey *et al*, 2001). Thus, MXR provides a potent new mechanism for MDR.

Finally, a recent study by Diah *et al* (2001) provided new evidence on the role of MRP in the emergence of MXR cross-resistance in breast cancer cell lines. They demonstrated that there was an increased MXR efflux in MRP1 overexpressing breast cancer cell lines probably due to a novel transport mechanism of glutathione-independent MRP1 substrates, since they found no evidence for involvement of GSH or other conjugates on MXR transport.

In conclusion, it is important to point out that the fact that multiple transporters can affect individual anticancer agents has significant implications for studies attempting reversal of drug resistance. As a consequence, there is overlapping substrate specificity of MDR, MRP and MXR as illustrated in **Figure 1.11**. Mitoxantrone and daunorubicin were accumulated in all MDR- , MXR- and MRP- expressing cells whereas drug accumulation profiles with known PgP substrates (bisantrene, topotecan, rhodamine 123 and prazosin) were expressed in MDR- and MXR- overexpressing cells (Litman *et al*, 2000). In contrast, the PgP substrate, vinblastin appeared not to be a good transport substrate for the MXR sublines whereas paclitaxel, non-fluorescent calcein-AM ester and colchicine were transported only from the MDR-overexpressing cell line (Litman *et al*, 2000 and Versantvoort *et al*, 1995). Consistent with this, a fluorescent analogue of the MDR reversing agent verapamil was clearly transported only from the MDR-overexpressing cell line (Litman *et al*, 2000). In contrast, the UV-excitable compound monochlorobimane was excluded only in the MRP-overexpressing cells, suggesting a

role of MRP as a glutathione S-conjugate pump, since monochlorobimane becomes fluorescent when bound to GSH (section 1.10) (Millis *et al*, 1997 and van Luyn *et al*, 1998). In addition, transport of calcein was only apparent in MRP-overexpressing cells (Litman *et al*, 2000). These observations highlight further both the complexity of MDR and the need for development of new and more specific inhibitors of clinical drug resistance.

*1.9.4* The lung resistant protein (LRP)

Lung resistant protein (LRP) is the 110 kDa human major vault transporter protein originally isolated from PgP-negative multidrug resistant cells. LRP is found in the cytoplasm and on the nuclear membrane and appears to function as a bi-directional nucleo-cytoplasmic transporter of molecules and particles. LRP has been shown to be overexpressed in a variety of cancer cells and is often co-expressed with MRP gene in highly multidrug resistant cells (Izquierdo *et al*, 1996; Ikeda *et al*, 1998).

The LRP has been identified to be overexpressed in ovarian cancer, acute myeloid leukaemia (AML) and multiple myeloma. Finally, LRP is widely expressed in normal human tissue, including peripheral blood and bone marrow.

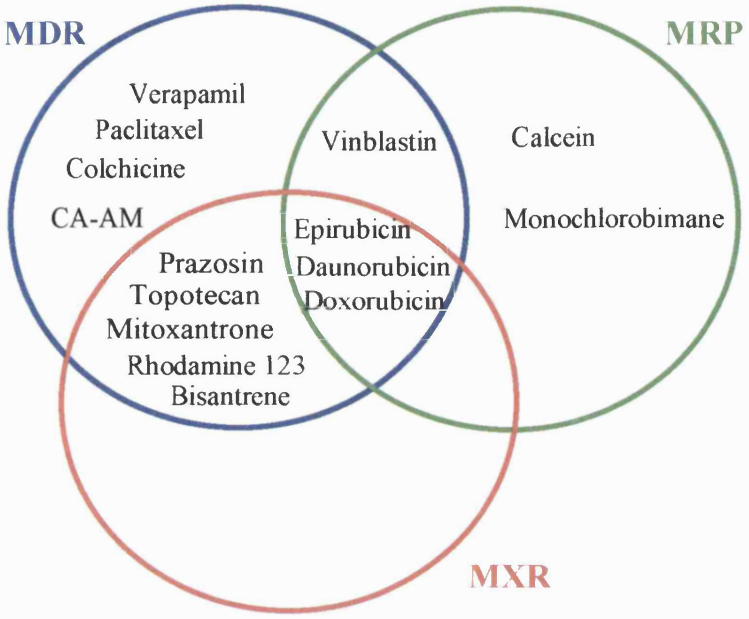



***Figure 1.11***: Diagrammatic representation of the substrate specificity of the ABC-transporters, MDR, MRP and MXR in cell lines overexpressing MDR, MRP and MXR, respectively (Adapted by Litman *et al*, 2000)

### 1.10 Evidence associating multidrug resistance and oxidative stress

The direct link between the MDR cells and resistance to oxidative agents is still controversial. Even so, it was primarily the work by Epsztejn *et al* (1999) that inspired us to study further the connection between MDR and oxidative stress. According to this study overexpression of H-Ft subunit in murine erythroid leukaemia cell line reduced the response of cells to oxidative stress by downregulating the level of the LIP in the cells and inducing MDR properties. In particular, the transient overexpression of H-Ft promoted a commensurate ability of cells to resist drug toxicity following oxidative challenges with $H_2O_2$. MDR properties of the H-Ft overexpressing cells involved increased levels of mdr1 mRNA and protein (PgP) as well as drug transport and drug toxicity.

There are also studies indicating that hyperferritinaemic patients suffering from eye cataract overexpress the L-Ft subunit and develop MDR properties within the epithelial cells of the eye (Dunia *et al*, 1996).

Recently, it has been suggested that an elevation of intracellular ROS is downregulating PgP expression via activation of receptor tyrosine kinase signalling pathways (Wartenberg *et al*, 2001). Downregulation of PgP was also observed with low concentrations of $H_2O_2$ and epidermal growth factor (EGF), indicating that ROS may regulate PgP expression.

Free radicals can also be involved in the cytotoxic effects of anticancer agents. This hypothesis was investigated by Mazzanti *et al* (1996), whose work was based on the study of the link between MDR phenotype and free-radical stimulated lipid peroxidation.

The authors demonstrated that the induction of the MDR phenotype in NIH 3T3 fibroblasts increases the susceptibility of cells to both irradiation and iron stimulated lipid peroxidation.

Anthracyclines such as doxorubicin have also shown to cause cytotoxicity by generating ROS, so it is possible that resistance to doxorubicin could be associated with increased intracellular enzyme capacity to convert ROS into active metabolites. In a study by Kim *et al* (2001), a doxorubicin-resistant AML subline that overexpressed MRP, but not PgP, although was found to be resistant to other drugs such as daunorubicin and vincristine, showed sensitivity to $H_2O_2$. This data suggested that decreased catalase activity could be attributed to MRP overexpression, which could pump out substances.

It is well established that the use of doxorubicin as an anticancer drug is limited because it shows severe cardiotoxicity. The relationship between iron metabolism and doxorubicin cardiotoxicity has been studied with cardiac biopsies and cardiomyocytes (Minotti *et al*, 1998 and 2001). Minotti *et al* (2001) demonstrated that doxorubicin irreversibly inactivates IRP-1 and IRP-2 in cardiomyocytes.

Moreover, in relation to UV studies, Trindale *et.al* (1999) have observed that leukaemia cells exhibiting MDR phenotype and overexpressing PgP, were resistant to UVA but not UVB or UVC, presumably as a result of higher catalase activity.

MDR has been shown to be associated with increased cellular levels of GSH and/or GST. It has been reported that elevated GSH levels in γ-GCSh transfected cells down-regulate MRP1 and γ-GCSh expression, indicating that these two genes may be co-ordinately regulated (Yamane *et al*, 1998). In addition, a study by Renes *et al* (2000)

indicated that MRP1 protects cells against the toxicity of lipid peroxidation products, specially the α,β-unsaturated aldehyde 4-hydroxynonenal (4HNE). GSH has a major role in the metabolism of 4HNE, since it can react with 4HNE using GST as a catalyst (**Figure 1.12**). Sometimes metabolites during GSH conjugation (GS-4HNE) become toxic by inhibiting GST activity. Such a conjugated product will then be transported across cell membranes via MRP1 efflux pump, leading to decreased intracellular concentration of toxins. Taken together, it appears that GS-4HNE is a new substrate for MRP1 and that MRP1, together with GSH, has a role in the defence mechanism against oxidative stress (see Renes *et al*, 2000).

Furthermore, a number of cell types have been shown to release GSSG via an MRP-mediated export mechanism. GSSG has been shown to be a substrate for MRP, suggesting that MRP by exporting GSSG compensates oxidative stress when GSSG becomes rate-limiting (Keppler, 1999). Also, Hirrlinger *et al* (2001) showed that $H_2O_2$-induced oxidative stress resulted in the production of GSSG and that its release was mediated by MRP.

It is well known that chemotherapeutic drugs such as doxorubicin, cisplastin and etoposide, cause apoptotic changes in the cell. It was initially a range of clinical studies that suggested that the expression of MDR genes involved in apoptosis affects chemosensitivity. Recent studies by Boland and co-workers (1997) showed that daunorubicin activates NFκB and induces kB-dependent gene expression in HL-60 and in Jurkat T lymphoma cells in association with the generation of ROS as a result of endogenous cellular processes that lead to apoptosis.

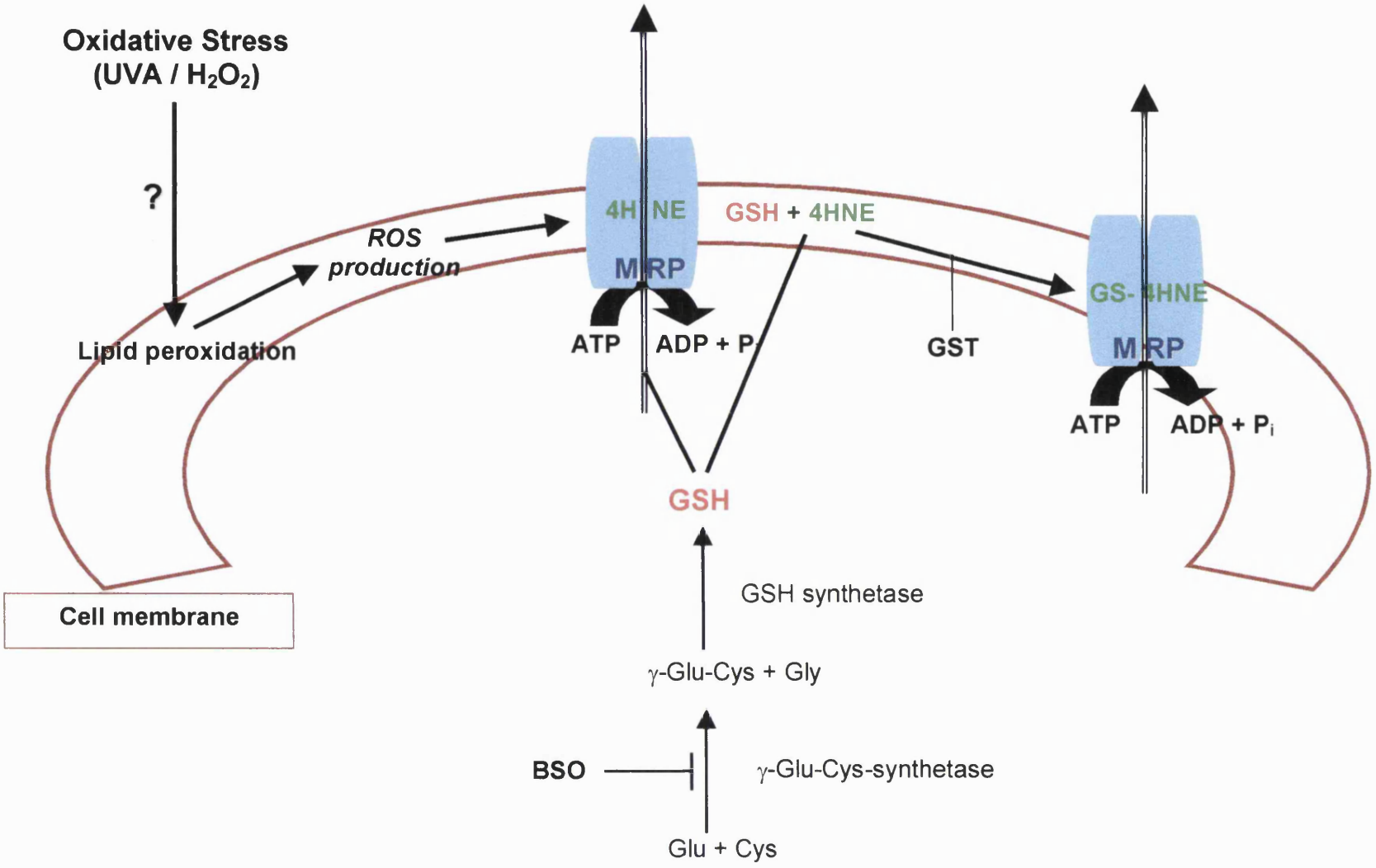



***Figure 1.12*:** Diagrammatic representation of the interrelation between MRP and GSH. Note that 4HNE is given as an example for this export mechanism of MRP and GSH.

Furthermore, studies by Màtarrese *et al* (2001) have also indicated that expression of PgP could sensitise lymphoblastoid CEM cells to mitochondria-mediated apoptosis. In particular, they demonstrated that Pgp function is associated with a sort of sensitisation to mitochondrially bound apoptotic stimuli (i.e. tumour Necrosis Factor (TNF-α)) and proposed that this mechanism might be associated with specific modification of the membrane potential (i.e. $\Delta\Psi$).

Although several studies have suggested that overexpression of the Apaf-1, required for mitochondria-dependent apoptosis, increases sensitivity to apoptosis induced by chemotherapeutic drugs, the first direct evidence was provided by Jia *et al* (2001) in human leukemic cells. They reported that constitutive variations in the levels of Apaf-1 protein in human leukemic cells could determine sensitivity to apoptosis downstream of mitochondrial involvement. Using UV light as a stimulus to pass through the mitochondrial barrier, they demonstrated that radiation treatment resulted in the release of cytochrome c from mitochondria in all tumour cells and that the differential susceptibility to cytochrome c dependent apoptosis was associated with the Apaf-1 protein level in these cells.

Besides these observations, further understanding of the physiological role of each of the MDR transporters is critical for determining their role and evaluating the consequences of alterations in their activity under conditions of oxidative stress.

## 1.11 Aim and objectives

Several recent studies (see section 1.10) have revealed that cells exhibiting MDR are also resistant to oxidizing agents including solar ultraviolet A radiation. However, the mechanisms underlying the appearance of such resistance are currently unknown. The objective of this project is to examine the functional link between MDR-associated proteins and resistance of cells to oxidizing agents such as $H_2O_2$ and UVA. To this regard we used a model of two human Jurkat T cell lines, one with non-resistance (parental) and one resistant to $H_2O_2$. Our primary goal was to characterise these cell lines in terms of susceptibility to oxidizing agents (i.e $H_2O_2$ and UVA) and investigate whether there is a correlation between LIP release and the susceptibility of cells to both oxidizing agents. Consequently our aim was to provide preliminary observations on correlation between the increased susceptibility of cells to oxidising agents and the level of expression of key MDR-gene products. Such studies are crucial for understanding of the mechanism of MDR and should provide evidence towards understanding of MDR expression and functionality in cancer cells.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Chemicals

All the reagents were from Sigma-Aldrich Chemical Co. (Poole, UK) unless otherwise specified. All the cell culture materials were obtained from Life Sciences Technologies (Paisley, UK), except the foetal calf serum (FCS), which was obtained from PAA (Austria). Dulbeco's Minimum Essential medium (DMEM) and Trizol were supplied by Gibco BRL Invitrogen Corporation (Life Technologies, UK). Calcein (CA), its acetoxymethyl ester (CA-AM) and the fluorescent dye, 3,3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$) were obtained from Molecular Probes (Leiden, Netherlands). Salicyladehyde isonicotinoyl hydrazone (SIH) was kindly provided by Prof. Ponka (Lady Davis Institute, Canada). Desferrioxamine mesylate (DFO) was from Novartis (Basel, Switzerland). Protease inhibitors and Annexin V were from Roche (Mannheim, Germany). HO-1 and HO-2 anti-human polyclonal antibodies were from Santa-Cruz Biotechnology. HO-1 monoclonal antibody was supplied from Stressgen Biotechnology, UK. Protein G-Sepharose and actin anti-mouse Ig, horseradish peroxidase (HRP), were from Amersham Pharmacia Biotech. ApoGlow Kit was supplied by Lumitech (UK).

## 2.2 Mammalian Cell Culture

The human Jurkat T cell lines (*J16* and *HJ16*) were kindly provided by Dr. Nick Hall (Pharmacy and Pharmacology, Bath University, UK).

***J16*:** The parental human Jurkat T cell line (stated as parental cells).

***HJ16*:** The $H_2O_2$ resistant Jurkat T cell line derived from the parental J16 following gradual adaptation to 3mM $H_2O_2$ (stated as $H_2O_2$ resistant cells).

*J16* and *HJ16* cells were cultured routinely and incubated in a humidified atmosphere at 37°C with 5% $CO_2$. Both cell lines were grown in RPMI-1640 medium, supplemented with 10% v/v FCS, 2 mM L-Glutamine, 50 IU/ml of each penicillin/streptomycin (P/S).

## 2.3 Treatments

*2.3.1* UVA irradiation

The broad spectrum 4kW lamp (Sellas, Germany) emits primarily UVA irradiation (significant emission in the range of 350-400 nm) and some near-visible radiation longer than 400 nm. The spectrum of the lamp is shown below in **Figure 2.1**:

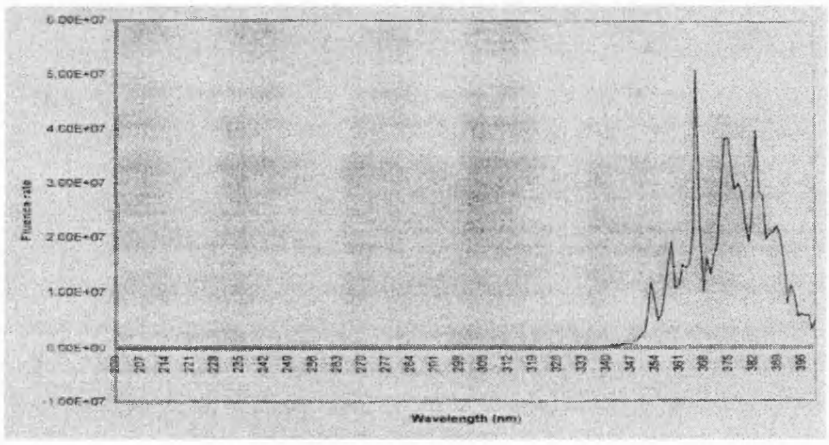



***Figure 2.1*:** Spectrum of the Sellas 4kW UVA lamp.

The UVA doses were measured using an IL1700 radiometer (International Light, Newbury, MA). Irradiation was carried out in an air-conditioned room at 18°C in order to maintain the temperature of the cells to approximately 25°C throughout the irradiation procedure.

Prior to irradiation, cells were washed with PBS and then PBS, containing 5ppm $Ca^{2+}$ and $Mg^{2+}$, was added to the cells in 6-cm plates. This was followed by irradiation of cells at 100, 250 and 500 $kJ/m^2$. Following the irradiation, the cells were resuspended in conditioned medium (the retained medium in which cells had been grown).

*2.3.2* Hydrogen peroxide ($H_2O_2$) treatment

Stock solution: 176 mM in PBS.

$H_2O_2$ treatment was carried-out in serum free media (SFM) in order to avoid any interference of catalase from the serum. The cells were incubated with $H_2O_2$ in SFM at the final concentrations of 0.05, 0.1, 0.5, 1 and 3 mM for 30 min at 37°C.

*2.3.3* Hemin and DFO treatments

Stock solutions: 20 mM hemin in dimethyl sulfoxide (DMSO)

150 mM DFO in $H_2O$

Prior to UVA and $H_2O_2$ treatments, cells were treated with hemin (20 μM) or DFO (100μM) for either 2 or 18h at 37°C.

*2.3.4* Anti-cancer drug treatments

J16 cells were treated with a range of doses of anti-cancer drugs in SFM.

Stock solutions: 1 mM of vinblastin, doxorubicin, mitoxatrone and verapamil in DMSO.

Dose range of vinblastin: 20, 50 and 70 nM

Dose range of doxorubicin: 5, 10, 20 and 50 μM

Dose range of mitoxatrone: 20, 40, 80 and 100 μM

**2.4 Flow Cytometry**

Quantification of apoptosis, by Annexin V- Fluoroscan isothiocyanate (FITC) staining, and necrosis, by Propidium Iodide (PI) uptake, were evaluated by flow cytometry. Annexin V is a phospholipid-binding protein with a high affinity to phosphatidylserine (PS), which during apoptosis translocates to the outer surface of apoptotic cells. Detection of cell surface PS with Annexin V serves as a marker for apoptotic cells. However, necrotic cells are taken up by simultaneous staining with both Annexin V and PI. Therefore, Annexin V and PI double-staining can differentiate between necrotic and apoptotic cells.

After the different incubation periods, cells were incubated with incubation buffer (10 mM Hepes/NaOH, pH 7.4, 5 M NaCl, 100 mM $CaCl_2$) containing Annexin V (20 μl/ml) and PI (20 μl/ml) for 15 min. Double stained cells were considered necrotic whereas Annexin V positive, PI negative cells were apoptotic. A minimum of 10,000 cells were analysed per sample on a FACSvantage (Becton Dickinson, Erembodegen, Belgium). The following settings were used for all the analysed cell lines: FL1 (530 ± 15 nm) in log data collection and FL3 in log data collection. The data was analysed using two-dimensional dot plots of FL3 versus FL1 fluorescent profiles. Data analysis was

performed with CellQuest software (Becton-Dickinson, Erembodegem, Belgium) and a dual parameter of FL1 (Annexin V) and FL3 (PI) was used.

### 2.5 MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl0-2,5-diphenyl tetrazolium bromide] assay was used in order to quantify the viability of the cells as well as cytotoxicity after treatments (Doyle and Griffiths, 1998). The principle of this assay is based on the capacity of intracellular dehydrogenase enzymes in living cells to convert the yellow-soluble substrate (MTT) in a dark blue formazan product that is water-insoluble. The amount of formazan produced is directly proportional to the cell number. Since a colorimetric assay, the analysis of results is performed using a micro-ELISA plate reader.

The procedure involves preparation of fresh MTT stock solution in serum free media (1ml of 5 mg/ml MTT diluted in 10 ml serum free media). After different post-incubation periods, cells were first diluted with MTT in SFM and then 200 µl of each sample was added in triplicates, in a 96-well microplate. Plate was incubated for 3h at 37°C. After incubation, MTT solution was aspirated and 100 µl of DMSO was added in each well. The plate was read in a 96-well microplate reader (Dynatech MR5000) at 550 nm.

### 2.6 Adenosine Triphosphate (ATP) Measurement

The ApoGlow screening kit is an effective research tool that differentiates between apoptosis and necrosis by detecting the ratio of ADP to ATP. Therefore, ATP concentrations in cells were determined luminometrically using the ApoGlow assay kit, as described by the manufacturer (Lumitech, UK). Cells were then treated with a range of

doses of $H_2O_2$. After $H_2O_2$ treatment cells were incubated with 150 µl of nucleotide releasing agent per sample and allowed to equilibrate at room temperature for 5 min. Twenty µl of nucleotide monitoring reagent was added and the first reading was recorded in the luminometer (Streptech) to determine ATP concentrations (i.e. **Figure 2.2**, reading A). After 10 min, 20 µl of ADP converting reagent was added and luminescence was read (reading B). The final reading was performed 5 min later (reading C). ADP concentrations were calculated as C-B. ADP/ATP ratios were calculated as (C-B)/A.

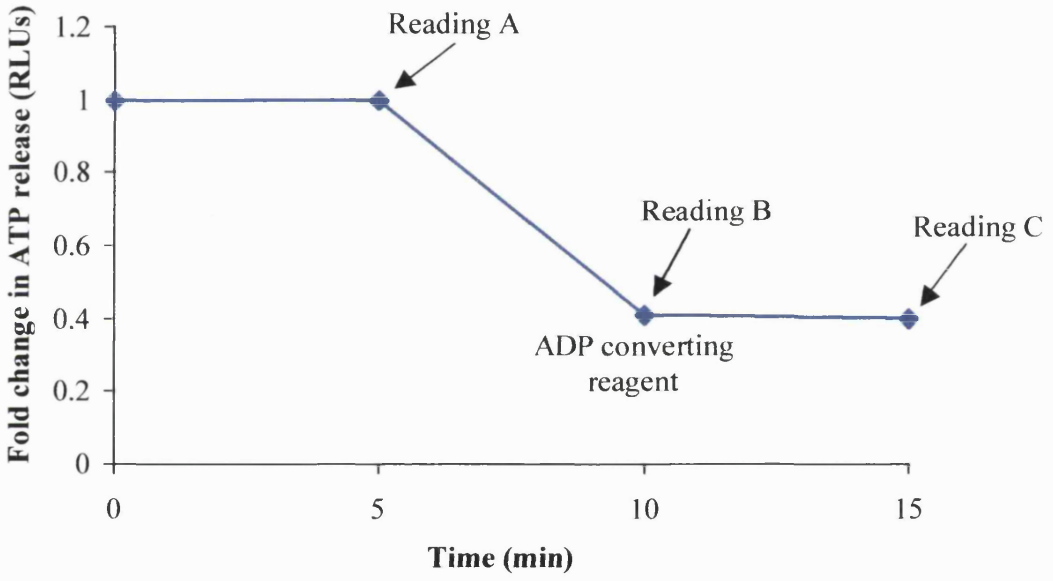



***Figure 2.2*:** Kinetics of the ApoGlow Kit. It illustrates an example of the fold change in the ATP levels.

### 2.7 LIP determination by Fluorescence calcein assay (CA-assay)

The level of LIP was estimated by a modification of the method developed by Epsztejn *et al* (1997). The principle of this assay is that the fluorescent CA, formed

intracellulary upon cellular loading with the nonfluorescent precursor CA-AM (**Figure 2.2**), binds to a fraction of the cellular iron associated with the LIP, thereby quenching its fluorescence. The level of intracellular CA-bound iron was determined by the increase in fluorescence produced by the addition of the iron chelator SIH, **Figure 2.2**). A schematic representation of the CA-fluorescent measurement in the spectrofluorometer is shown in **Figure 2.3**.

Immediately after UVA or $H_2O_2$ treatments cells were loaded with 0.05 μM calcein-AM in 1 ml Earle's minimum essential media (containing 20 mM HEPES, pH 7.3) for 15 min at 37°C. Then cells were washed with PBS and resuspended in 10 mM HEPES buffer containing 2 mM diethyltriamine-pentaacetic acid. The cell suspension was transferred to a thermostatically controlled cuvette and CA fluorescence was monitored on a spectrofluorimeter with excitation at 480 nm and emission at 517 nm. The level of intracellular calcein-bound iron (CA-Fe) was measured by the increase in fluorescence signal after the addition of SIH (40 μM, final concentration).

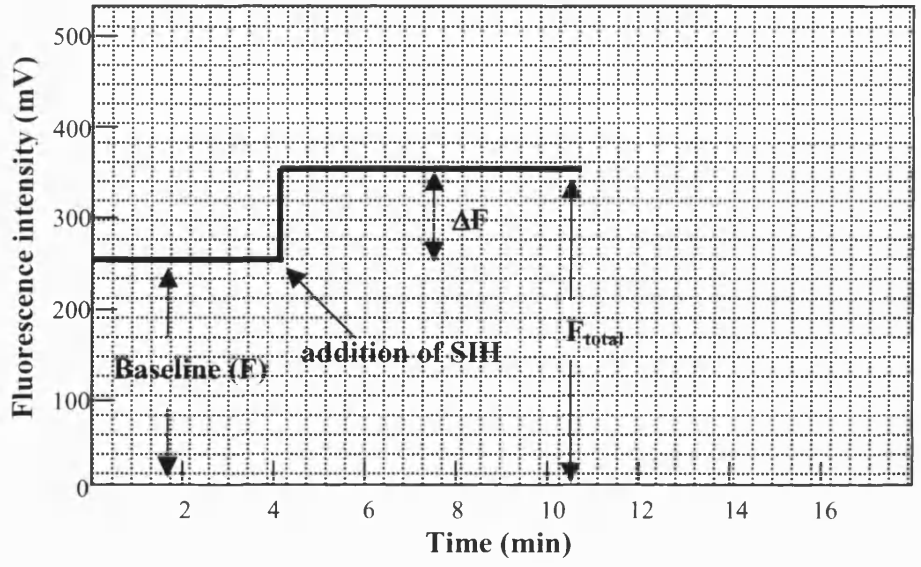
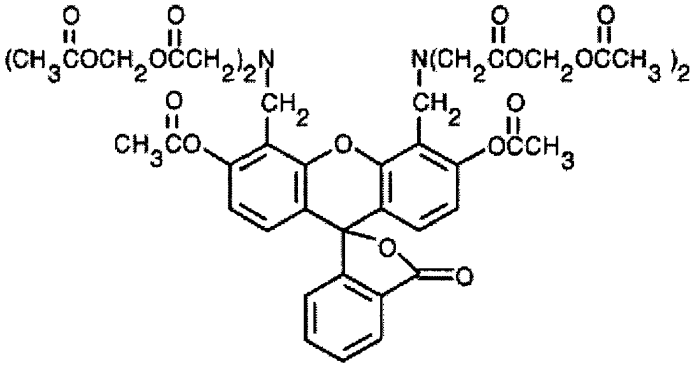



***Figure 2.3***: Schematic representation of the change in fluorescence after the addition of the iron chelator, SIH. F represents free CA, ΔF represents CA-Fe and $F_{total}$ is the total fluorescence representing free CA + CA–Fe.

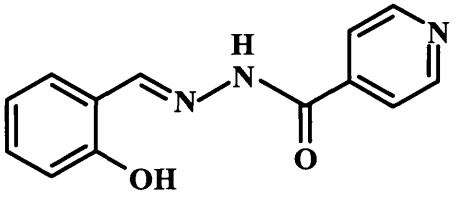
In order to obtain the relationship between the changes in fluorescence elicited by SIH and LIP in cells, calibrations were performed in untreated cells. Firstly, the relationship between fluorescence intensity of CA and intracellular iron concentration was performed by titration of CA-loaded cells via the addition of ferrous ammonium sulphate (FAS). Since FAS is a source of $Fe^{2+}$, a divalent metal ionophore A23187 was added to internalise the compound. After treatment with 10 μM A23187, 0.1 μM FAS was added cumulatively and the corresponding change in fluorescence was determined.

Secondly, the determination of dissociation constant (Kd) of calcein-bound iron was also required so that the free iron concentration in the cells could be calculated. The dissociation constant was calculated with a 5 μM CA solution and was based on the titration of CA-loaded cells with FAS. From the titration, the free CA fluorescence intensity was first normalised to the initial fluorescence value and then plotted against the metal concentration.

Finally, the cell volume was also considered. Cell volume was measured by using a certain amount of cell suspension ($12 \times 10^6$). The cells were resuspended in 200 μl PBS, transferred into the capillary tubes, sealed with Cristasel, and centrifuged at 1000 rpm for 1 min in a Hameatocrit centrifuge GIBA-GEIGY TH12. The cell volume was calculated by using the percentage of cell packed volume divided by the number of cells.



(A): Calcein (CA)



(B): Salicylaldehyde isonicotinoyl hydrazone (SIH)

**Figure 2.2**: Chemical structures of calcein (A) and SIH (B).

## 2.8 Ferritin ELISA

The level of Ft after different treatments was determined using a polyclonal (anti-ferritin) enzyme-linked immunosorbent assay (ELISA) kit known as Enzyme-Test® Ferritin kit (Roche, UK). For this assay, cytosolic extracts are prepared and added to a streptavidin tube along with biotinylated monoclonal, mouse anti-Ft antibodies, labelled with peroxidase (the incubation solution). The antibodies bind to streptavidin, thus anchoring the ferritin/antibody complexes to the tube. Next, a peroxidase substract, ABTS® (di-ammonium 2, 2'-azino bis 3-ethylbenzothiazoline-6-sulphonate), is added and is cleaved by the peroxidase to produce a characteristic green colour, which is measured spectrophotometrically at 420 nm. Ferritin levels were expressed as ng ferritin per mg of protein.

### *2.8.1* Preparation of cytosolic extracts

Cells were collected at $3x10^6$ cells/ml after the treatments and washed in ice-cold PBS. The cells were re-suspended in 1x Munro lysis buffer [10 mM Hepes at pH 7.5, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, chymostatin (50 µg/ml) and 0.3% NP-40 (Merck, UK)] and flash-frozen in dry-ice with 96% Ethanol. Cell extracts were then stored at -70°C till use.

### *2.8.2* Protein measurement

Cytosolic protein concentration was determined by Bradford assay with the BioRad reagent kit (Bradford, 1976). A series of concentrations of BSA (100 mg/ml, stock) were made from 0 to 25 µg/ml for the calibration of standard curve and absorbance

measurements obtained using a 96-well microplate reader (Dynatech MR5000) at 595 nm. Measurements were performed in duplicate on 10 µl aliquots of the extracts and the mean values were obtained from duplicate experiments.

*2.8.3* Ferritin analysis

Ferritin standards and cell extracts were added to streptavidin-coated tubes with the incubation buffer (containing anti-ferritin antibodies) and incubated at room temperature (RT) for 30 min. The tube contents were then aspirated and rinsed with washing solution. The chromogen substrate solution (ABTS®) was then added to each tube and incubated for 15 min at RT. The absorbance was measured immediately at 420 nm using the spectrophotometer (UNIKON 922, Switzerland). The Ft concentration in each extract was determined against the ferritin standards and normalised using the total cytosolic protein concentration obtained from the Bradford assay.

**2.9 Metabolic labelling in cells**

Biosynthesis of Ft protein under control, hemin or DFO stimulated and UVA- or $H_2O_2$- treated conditions was evaluated by means of metabolic labelling using [$^{35}$S]methionine. Briefly, 2 x $10^7$ cells per sample per time-point were starved for 30min in methionine-free minimum essential media supplemented with 5% FCS followed by [$^{35}$S]methionine labelling (7 µCi/ml) for 1h 30min. The cells were then treated with either UVA or $H_2O_2$, depending on the conditions, apart from hemin and DFO treatments in which hemin or DFO were always present, during starvation and labelling of the cells. Cells were then lysed in immunoprecipitation buffer (50 mM Tris, pH 7.4 / 150 mM

NaCl / 10 mM EDTA / 1% Triton X-100 / 1% SDS / 1x cocktail of protease inhibitors / 20 μg/ml leupeptin and 100 μg/ml chymostatin). The efficiency of metabolic labelling was monitored by trichloroacetic acid (TCA) precipitation, since it is used for monitoring the incorporation of radioactivity into cellular proteins. Equal amounts of labelled proteins were then immunoprecipitated by using either anti-human ferritin polyclonal antibody, followed by Protein G-sepharose and loaded on a 12% SDS-polyacrylamide gel. Gel run overnight at 40 V and then fixed with 100 ml isopropanol, 40 ml acetic acid and 260 ml water for 30 min and dried. After drying, the gel was exposed for autoradiography.

**2.10 Western blotting**

Western blotting was used for the identification and semi-quantification of specific proteins, which have been separated according to their size by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). After different treatments and post-incubation times, cells were lysed with 1 x Munroe lysis buffer (10 mM Hepes pH 7.5, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 0.3% NP40, 50 μg/ml pefablock and MilliQ water to get 1 ml final volume per 3 x $10^7$ cells). Protein measurement was then determined as described in section *2.8.2.*

Equal amounts of protein (30 μg per condition) were diluted with 3 x loading buffer [180 mM Tris, pH6.8 / 3% SDS / 150 mM DTT (1,4-dithio-DL-threitol) / 30% glycerol / 0.0015% bromophenol blue / 0.53 ml milliQ water] and boiled for 5 min. Proteins were separated on a 10% polyacrylamide gel and electrotransferred onto nitrocellulose membranes using BioRad electro-transfer unit. Membranes were stained with red

ponceau c to ensure equal loading and transfer of protein. Membranes were then blocked for 1h 30 min (room temperature) with 5% milk powder in 0.05% Tween-PBS for HO-1 antibody and with 3% milk powder, 3% BSA in 0.1% Tween-PBS for actin antibody. After blocking, membranes were incubated overnight (4°C) with 1:1000 polyclonal HO-1 antibody (goat polyclonal IgG) or for 1h 30 min (room temperature) with 1:500 monoclonal actin antibody (produced in mouse). After incubation period of primary antibody, membranes were washed with Tween-PBS for three times every 10 min. Membranes were then incubated with 1:1000 anti-goat IgG HRP antibody for HO-1 or 1:2500 anti-mouse Ig HRP antibody for actin, for 1h at room temperature. Immunoreactive bands were visualised with the enhanced chemiluminescence detection system by Amersham Biosciences.

**2.11 Measurement of HO-1 expression by Flow cytometry**

Flow cytometric analysis was used for the detection of expression of HO-1 in both control and hemin stimulated parental and $H_2O_2$ resistant cells. Briefly, 1 x $10^6$ cells were treated with hemin for different time points (2, 4, 6 and 8 hours). After treatment cells were fixed in 1 ml of 70% ice-cold ethanol. Cells were blocked with 500 ml bovine serum albumin (BSA) for 10 min at 37°C. After blocking, cells were incubated with mouse anti-HO1 monoclonal antibody in the dark for 60 min. After incubation of primary antibody cells were washed twice with PBS and incubated with an anti-mouse FITC antibody in dark for 60 min. Cells were then washed twice with PBS and resuspended in 300 µl PBS for flow cytometric analysis. A minimum of 10,000 cells were analysed per sample on a FACSvanatge (Becton Dickinson, Erembodegen, Belgium). The following

settings were used for all the analysed cell lines: FL1 (530 ± 15 nm) in log data collection. The data were analysed using FL1 fluorescent profiles. Data analysis was performed with CellQuest software (Becton-Dickinson, Erembodegem, Belgium).

**2.12 Catalase activity assay**

Catalase activity in both normal and $H_2O_2$ resistant cells was quantified by a modification of the method developed by Moysan *et al* (1993). This assay is based on the direct decomposition of $H_2O_2$ to ground state oxygen and water without the use of another substrate as GPx does. Therefore, catalase activity is estimated by the direct measurement of $H_2O_2$ consumption.

Cells (1 x $10^6$) were lysed in 225 µl of phosphate buffer (50 mM $KHPO_4$, pH 7.5), to which 25 µl Triton X100 (0.1% final concentration) was mixed. The suspension was sonicated on ice for 60 sec at high strength (Branson B-12 sonifier,Branson Sonic Power, Danbury, CT) and spun at 13000 rpm for 3 min. The clear supernatant was collected and used for the assay. Next, 100 µl of the lysate was mixed at 25°C with 600 µl $H_2O_2$ (10 mM solution in phosphate buffer). Hydrogen peroxide consumption was then monitored spectrophotometrically at 240 nm using a thermostatically controlled quartz cuvette (25°C) for 1 to 2 min. Catalase activity was normalised to sample protein content (protein was measured by Bradford assay, see *2.7.2*) and expressed as Unit per mg (U/mg) protein. One unit (U) is defined as 1 µmole $H_2O_2$ consumed per min.

## 2.13 Glutathione peroxidase (GPx) activity assay

The GPx activity in both normal and $H_2O_2$ resistant cells was measured according to the method developed by Flohe and Gunzler (1984), with a few modifications. The principle of this assay is that GPx metabolises $H_2O_2$ by reduction of GSH resulting in the formation of water. The oxidised glutathione (GSSG) is reduced to GSH by glutathione reductase (GR) in the presence of NADPH. Thus, GPx activity is determined spectrophotometrically by the decrease in NADPH concentration.

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

$$GSSG + H^+ + \mathbf{NADPH} \xrightarrow{GR} 2GSH + \mathbf{NADP^+}$$

Cells ($1x10^6$) were lysed in 500 µl of buffer (100 mM tris-HCl, 300 mM KCl, 0.1% peroxide-free Triton X100, pH 7,6) by sonication. The cells were pelleted at 4000 rpm for 10 min and the clear supernatant was collected. A test mix containing 100 mM Tris-HCl (pH 7.6), 5 mM EDTA, 3 mM GSH, 0.2 mM NADPH, 0.1% Triton X100, 600 mU/ml GR, was prepared and preincubated at 37°C for 10 min. The reaction started by the addition of 10 µl tert-butyl hydroperoxide (50 µM final concentration) and the NADPH consumption rate was spectrophotometrically measured at 340 nm for 5 min. GPx activity was normalised to sample protein content (protein was measured by Bradford assay, see *2.7.2*) and expressed as U/mg protein. One unit was defined as 1 µmole NADPH oxidised per min.

## 2.14 Glutathione (GSH) measurement

Glutathione is conveniently assayed by an enzymatic recycling procedure where it is sequentially oxidized by DTNB (Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid))

and reduced by NADPH, in the presence of GR (Tietze, F. 1969). The resulting product 2-nitro-5-thiobenzoic acid (chromogenic) was spectrophotometrically measured at 412nm for 6-10 min and the glutathione present in cells is evaluated by comparison of the resulting values with the standard curve. This assay determines both GSH and GSSG.

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

For the standard curve, GSSG stock solution is diluted in 5% TCA / 2 mM EDTA in a range of 0.01 – 2 µM. For the samples, after treatment of cells with 50 µM of BSO, cell pellets were resuspended with 5% TCA / 2 mM EDTA in order to give 1ml of extract per $2 \times 10^6$ cells.

For the assay, 1 ml phosphate-buffer EDTA was mixed with 20 µl DTNB (1.5 mg/ml in 0.5% $NaHCO_3$), 50 µl NADPH (5 mM in 0.5% $NaHCO_3$) and 100 µl samples from either treated cells or GSSG samples for the standard curve in an eppendorf tube and transferred to a thermostatically controlled cuvette at 25°C. To the prewarmed mixture, 100 ml GR (18 u/ml in phosphate buffer) was added and the change in absorbance was monitored in the spectrophotometer for 6-10 min at 412 nm (25°C).

**2.15 Total RNA preparation**

Total RNA was extracted from control, $H_2O_2$ and UVA treated samples using Trizol. Cell pellets were treated with chloroform/isoamyl alcohol (49:1) in order to separate the organic and aqueous phase, which contains the nucleic acids. Aqueous phase was then transferred to a fresh eppendorf tube and an equal volume of isopropanol was added to the tube for precipitation. Samples were then centrifuged and the RNA pellet was washed with 75% cold-ethanol and left to dry. The RNA pellet was re-suspended in RNase-free

water and RNA measurement was carried out spectrophotometrically at 260 nm and the calculations were performed according to the following equation:

Absorbance $_{260}$ x 201 (dilution factor) x 40 = RNA concentration in μg/ml.

*2.15.1* Reverse transcriptase-PCR (RT-PCR)

Reverse transcriptase-PCR was performed for each RNA sample using the GeneAmp PCR system 2400 for first strand cDNA synthesis. Both RT-positive and RT-negative samples were prepared containing 1 μg total RNA in a final volume of 8 μl with water. Only the RT-positive samples were containing 2 μl of 50 μM poly $d(T)_{12-18}$ to give a final volume of 5 μM. The reaction mix used for RT-PCR is shown in **Table 2.1**. Reaction components for RT-positive and RT-negative samples were set-up separately. RT-positive samples were denatured in PCR machine for 10 min at 70°C and chilled immediately on ice. After that 8 μl of the reaction mix was added in all samples (RT-positive and RT-negative) followed by reverse transcription at 42°C for 60 min, then 95°C for 5 min and held at 4°C. The newly synthesised cDNA was stored at -80°C till used.

*2.15.2* Polymerase chain reaction (PCR)

The aim of this step is to use the PCR primers to amplify the gene of interest. For each target cDNA one master mix was prepared (shown in **Table 2.2**) that contained one set of specific forward ($P_1$) and reverse ($P_2$) primers. In this study, the housekeeping gene β-actin was used as control to estimate the amount of HO-1, mxr, mdr-1 and mrp-1 cDNAs in each sample. β-actin primers ($P_1$: 5' CAT CAC CAT TGG CAA TGA GC 3'

and $P_2$: 5' ATA CTC CTG CTT GCT GAT CC 3'), HO-1 primers ($P_1$: 5' CCT TGT TGA CAC GGC CAT GAC CAC 3' and $P_2$: 5' AGT TAG ACC AAG GCC ACA GTG CCG 3'), mxr primers ($P_1$: 5' TGC CCA GGA CTC AAT GCA ACA G 3' and $P_2$: 5' GAC TGA AGG GCT ACT AAC C 3'), mdr-1 primers ($P_1$: 5' CAG ACA GCA GCT GAC AGT CCA AGA ACA GGA CT 3' AND $P_2$: 5' GCC TGG CAG CTG GAA GAC AAA TAC ACA AAA TT 3') and mrp-1 primers ($P_1$: 5' CGG AAA CCA TCC ACG ACC CTA ATC C 3' and $P_2$: 5' TCC ACC TCC TCA TTC GCA TCC ACC TTG G 3') were used to amplify cDNAs obtained from the same total RNA. PCR programme was set depending on the primers used, as detailed below:

- β-actin: initial denaturation step 5 min at 94°C, then 30 cycles of 30 sec at 56°C (annealing step) and 1 min at 70°C (elongation step). Final elongation step 7 min at 72°C and held at 4°C.
- HO-1: initial denaturation step 5 min at 95°C, then 35 cycles of 30 sec at 52°C (annealing step) and 1 min at 72°C (elongation step). Final elongation step 7 min at 72°C and held at 4°C.
- mdr1: initial denaturation step 5 min at 95°C, then 30 cycles of 45 sec at 95°C (denaturation step), 1 min at 52°C (annealing step), 1 min and 30 sec at 72°C (elongation step). Final elongation step 7 min at 72°C and held at 4°C.
- mxr: initial denaturation step 5 min at 95°C, then 30 cycles of 30 sec at 95°C (denaturation step), 30 sec at 55°C (annealing step) and 3 min at 72°C (elongation step). Final elongation step 7 min at 72°C and held at 4°C.

- mrp1: initial denaturation step 5 min at 95°C, then 30 cycles of 45 sec at 95°C (denaturation step), 1 min at 55°C (annealing step), 3 min at 72°C (elongation step). Final elongation step 7 min at 72°C and held at 4°C.

PCR amplified DNA products were evaluated by agarose gel electrophoresis (section *2.15.3*). In order to check for genomic contamination RT-negative samples were used and a water control was used for any possible DNA contamination. For the quantification of the mxr and mdr-1 genes cDNA probes were used as positive controls at a copy range of $4.4 \times 10^6$ (MWG Biotech, UK).

*2.15.3* Agarose Gel Electrophoresis

Gel electrophoresis was performed as described by Sambrook *et al* (1989). For fine separation and analysis of DNA, 2% agarose with 1 mg/ml ethidium bromide was loaded with 15 µl of each PCR sample, diluted with 5x loading buffer [300 mM Tris, pH6.8 / 5% SDS / 250 mM DTT / 50% glycerol / 0.0025% bromophenol blue / 0.88 ml milliQ water]. Gel was run with 0.5x TBE (for 1 litre: 54 g Tris-base, 27.5 g boric acid and 20 ml 0.5 M EDTA, ph 8.0) buffer at 100 volts for 2 hours. The samples were then visualised using UV translluminator and the GeneGenius gel documentation and analysis system (Syngene Ltd).

| Reactants | Stock | Amount | Final Concentration |
|---|---|---|---|
| **RT Buffer** | 5 x | 4 µl | 1 x |
| **DTT** | 100 mM | 1 µl | 5 mM |
| **dNTP mix** | 10 mM | 1 µl | 500 µM |
| **Rnasin** | 50 U/µl | 1 µl | 2.5 U/µl |
| **Superscript II** | 200 U/µl | 1 µl | 10 U/µl |

**Table 2.1:** Protocol for the RT-PCR reaction mix of total RNA using Superscript II.

| Reactants | Stock | Amount | Final Concentration |
|---|---|---|---|
| **PCR buffer with $Mg^{2+}$** | 10 x | 2.5 µl | 1 x |
| **dNTP mix** | 10 mM | 0.6 µl | 200 µM |
| **Expand polymarase** | 3.5 U/µl | 0.15 µl | 0.05 U/µl |
| **$P_1$ + $P_2$ primers** | 5 µM | 3 µl + 3 µl | 500 nM |
| **cDNA** | - | 2 µl | - |
| **Water** | - | 13.75 µl | - |

**Table 2.2:** Protocol for the PCR reaction mix using expand polymerase.

## 2.16 Real time PCR – Light Cycler®

Light cycler real time PCR system (Roche Molecular Biochemicals, Germany) is an extremely powerful tool for quantitative PCR with the use of a double-stranded (ds) DNA binding dye, SYBR Green I (Roche Molecular Biochemicals, Germany), which fluoresces only when bound to dsDNA. The fluorescence is recorded by the end of the elongation phase at 530 nm and increasing amounts of PCR product are monitored. In addition, determination of the melting temperature ($T_m$) of PCR products with SYBR Green I confirms product identity and differentiation from non-specific products such as primers-dimers. Therefore, real time PCR was used to amplify HO-1 using β-actin as control.

All reactions were carried out in a total volume of 20 μl. Each reaction mixture contained 2 μl of 10x SYBR Green I dye, 2 μl cDNA, 25 mM $MgCl_2$, 5 μM of both forward and reverse primers and water to make-up to 20 μl. For the amplification step the conditions were the same with PCR, see section *2.14.2*, except from the cycle number that it was set to be 40 for the Light cycler conditions. Melting curve analysis consisted of 1 cycle of 95°C with 0 sec hold, 65°C with 15 sec hold and 95°C with 0 sec hold and continuous acquisition mode.

For quantification of each PCR product, a standard curve (i.e. **Figure 3.3**) was produced using a range of different concentrations of β-actin and HO-1 positive cDNA samples ranging from 1 to 10 ng of cDNA.

The real-time fluorescence signal generated by the SYBR Green I dye was quantified

in the analysis screen of the light cycler software. The software used the intersection between each fluorescence curve and a crossing line, to give a value for a crossing point. The crossing point valued for a set of standards and was plotted to give a standard curve. This standard curve was used to determine the starting template concentration of the unknown samples.

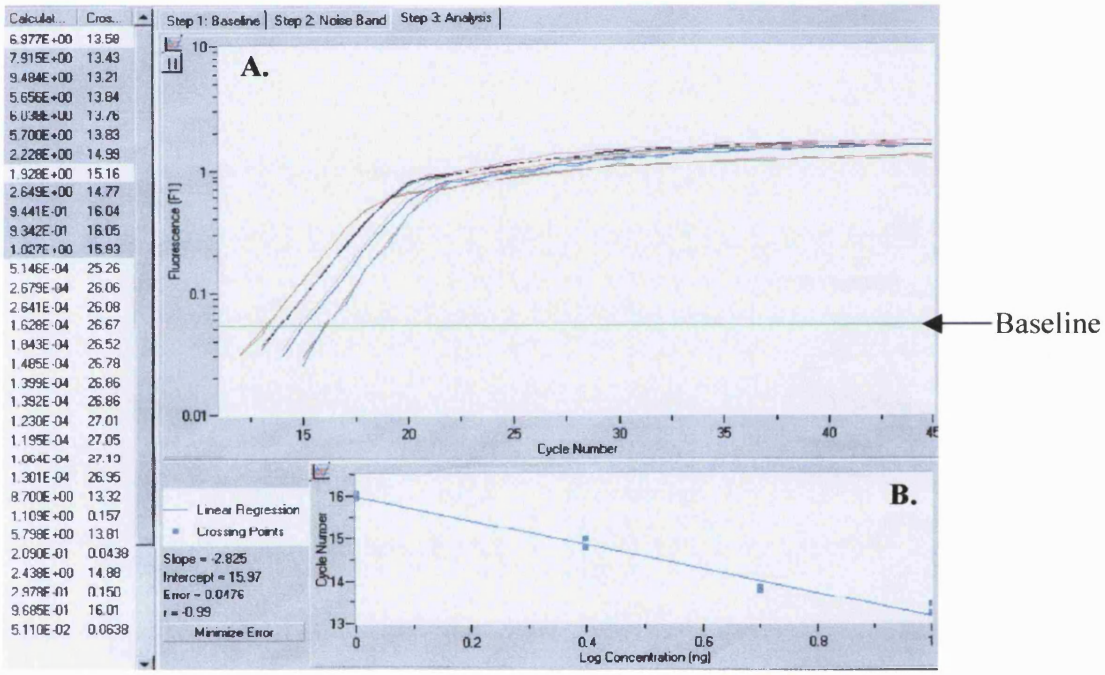



***Figure 3.3*:** Representation of the Light Cycler® software for the analysis and calculation of the concentrations of the gene of interest in different samples. The software calculates the concentration of target molecules by plotting logarithm of fluorescence versus cycle number and setting a baseline x-axis (A). The baseline identifies the cycle in which the log-linear signal can be distinguished from the background for each sample (A). The x-axis crossing point of each standard is measured and plotted against the logarithm of concentration to produce standard curve (B). The concentration of target sequence in the samples are extrapolated from the standard curve.

## 2.17 Flow Cytometric detection of functional dye/drug efflux

The modulation of drug efflux by 10 μmole/L verapamil was studied on flow cytometry using the fluorescent dye 3,3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$), an established substrate for PgP.

Normal and $H_2O_2$-resistant cells were resuspended with Dulbeco's Minimun Essential medium (DMEM), without phenol red, supplied with 10% FCS. Five samples per cell line were prepared: first sample (resuspended in PBS) was kept on ice as a control, rest of the samples ($1 \times 10^6$ cells per sample) were resuspended with 1 ml DMEM + 10% FCS containing 2 μl of 30 μg/μl $DiOC_2(3)$ and only one of the samples had 10 μl of 1 mM verapamil. All samples were incubated for 30 min at 37°C (dark conditions). After incubation, samples were washed with ice cold PBS and the two samples with $DIOC_2(3)$ and $DIOC_2(3)$ + verapamil, were kept on ice for immediate measurement of baseline $DIOC_2(3)$ uptake. The rest two of the samples were resuspended with 1 ml DMEM + 10% FCS containing 2 μl of 30 μg/μl $DiOC_2(3)$ and only one of the samples had 10 μl of 1 mM verapamil. Samples were incubated for 90 minutes at 37°C to allow time for active efflux to occur. After efflux, cells were washed and resuspended in PBS. As a positive control for this study, a human T-lymphoblastic CEM and its vinblastine-resistant subclone $CEM/VLB_{100}$ (Beck *et al*, 1979, Foley *et al*, 1965) leukemia cell lines were used.

$DiOC_2(3)$ is excited at a wavelength of 488 nm and emits at 500 nm. Therefore, fluorescent intensity of $DiOC_2(3)$ was detected on fluorescence channel one (FL1) of the FACScan.

## 2.18 Statistical analysis

Results are expressed as mean ± standard deviation (SD). Paired or unpaired Student's one-tailed *t*-test was used as appropriate to test differences between groups of data. Note that the rejection p value is 0.05. All analyses were carried out using Microsoft Excel and Origin 6.0.

# CHAPTER 3

# RESULTS

### 3.1 Effects of $H_2O_2$ treatment and UVA radiation on the cell survival

Hydrogen peroxide is toxic to most of the cells and, as a ROS, is involved in propagation of cellular damage due to its reactivity towards lipids, proteins and DNA (e.g. Brunk *et al.*, 1995). The major intracellular anti-oxidant enzymes catalase and GPX usually act upon $H_2O_2$ to either directly break it down to water and oxygen or to convert it to water and alcohol via oxidation of GSH (see Section 1.6).

UVA, the oxidizing component of sunlight is also harmful to cells as it generates ROS in cells via interaction with intracellular chromophores promoting biological damage in exposed tissues (Black, 1987; Trenam *et al.*, 1992; Yasui *et al.*, 2000). Several studies have demonstrated that both non-enzymatic and enzymatic anti-oxidant systems could significantly decrease the oxidative stress generated by UVA (see Tyrrell, R.M. 1991 and 1996).

However if cells are exposed to a severe oxidative insult, the anti-oxidant defence mechanism often does not suffice to counteract the oxidative imbalance in cells and as a result cells will die by either of two distinct mechanisms, necrosis (unordered and accidental form of cellular dying) or apoptosis (programmed and managed form of cell death). It has also been observed in some cases that an oxidizing insult could promote simultaneously both apoptotic and necrotic cell death (see Reed *et al*, 1999

and unpublished data, this laboratory). Since apoptosis and necrosis are considered conceptually and morphologically distinct forms of cell death, it is not clear how the same initial insult decides the prevalence of either phenomenon. Recent unpublished data from this laboratory provide strong evidence that UVA-induced immediate increase in LIP reflects the extent of necrotic cell death in both human skin fibroblasts and keratinocytes. Since exposure of skin cells to $H_2O_2$ also promotes labile iron release, it is reasonable to suggest that this phenomenon may also be linked to increased susceptibility of cells to necrotic cell death.

Our cell model of parental and $H_2O_2$ resistant Jurkat T cells provided us with an opportunity to investigate further these phenomena by first looking at how intracellular anti-oxidant defence mechanisms might play a role in adapting cells to a source of oxidative stress. Next, the possible role of labile iron, heme, Ft and HO-1 in increasing the resistance of cells to $H_2O_2$ was investigated.

Sections **3.1** to **3.8** provide an in depth comparison of these parameters between the parental and $H_2O_2$ resistant cell lines following treatment with the oxidizing agents $H_2O_2$ and UVA radiation.

*3.1.1* Determination of the level of $H_2O_2$ resistance

To evaluate the level of resistance of both parental and $H_2O_2$ resistant cells to $H_2O_2$, the level of apoptotic and necrotic cell death were scored by flow cytometry using the dual Annexin V/PI staining method (see section 2.4). For this purpose, cells were first treated with a range of doses of $H_2O_2$ and then incubated for either 4 or 24 h at 37°C prior to analysis by flow cytometry. The results of 4 h post-treatment revealed that in cells treated with low doses of $H_2O_2$ (i.e. up to 0.5 mM) no significant difference in cell survival could be observed between the parental and $H_2O_2$ resistant

cell line (**Figure 3.1**). However, at higher doses of $H_2O_2$ (i.e. 1 and 3mM), the percentages of live cells were significantly higher in $H_2O_2$ resistant cells when compared to parental cells. The higher resistance of $H_2O_2$ resistant cells to $H_2O_2$ became even more apparent when the treated cells were incubated for a longer period (i.e. 24 h post-treatment) prior to flow cytometric analysis. Indeed, we observed that 24 h following $H_2O_2$ treatment, the percentage of live cells drops dramatically in parental cells when compared to $H_2O_2$ resistant cells (**Figure 3.2**). In particular, the difference in percentage of live cells between 0.5 to 1 mM $H_2O_2$ treated parental and $H_2O_2$ resistant cells was significant (†, $p<0.05$). In parental cells, the drop in percentage of live cells coincided with a reciprocal increase in the percentage of necrotic cells. On the other hand, in $H_2O_2$ resistant cells, necrosis occurred to a lesser extent when compared to parental cells, confirming the resistance of cells to $H_2O_2$ up to final concentration of 1 mM. The difference in the percentage of necrotic cells between the two cell lines (*, $p<0.05$) was also statistically significant. Interestingly, the level of apoptosis was low in both cell lines, indicating that necrosis is the primary mode of cell death induced by $H_2O_2$.

*3.1.2* Effects of UVA on the induction of cell death

To examine whether cells resistant to $H_2O_2$ are also resistant to UVA radiation, both cell lines were treated with UVA doses of 100, 250 and 500 kJ/m$^2$. After UVA radiation cells were incubated for either 4 or 24 h in conditioned media and then the level of apoptotic and necrotic cells were scored by flow cytometry. The results showed that at both 4 (**Figure 3.3**) and 24 h (**Figure 3.4**) post-UVA treatment, there was no significant difference in cell survival between parental and $H_2O_2$ cell lines. However in both cell lines a dose-dependent increase in necrotic cell death was

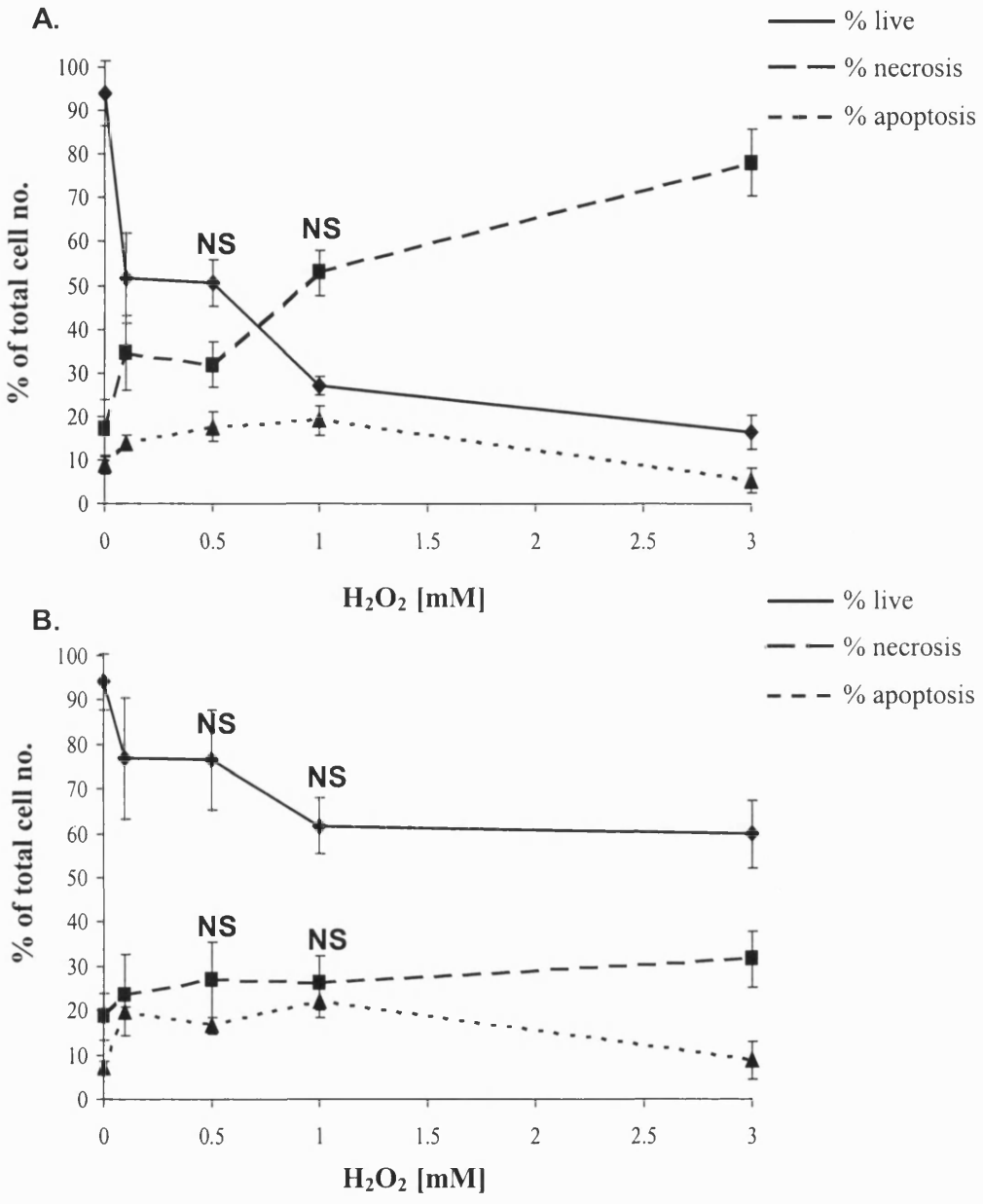



***Figure 3.1:*** Effect of different $H_2O_2$ doses on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells.
Note: Cells were incubated for 4 h post-treatment prior to dual Annexin V / PI staining and flow cytometric analysis. Mean ± SD (n = 3)
NS : $p > 0.05$, no significant difference between parental and $H_2O_2$ resistant cells at corresponding $H_2O_2$ doses in live and necrotic cells.

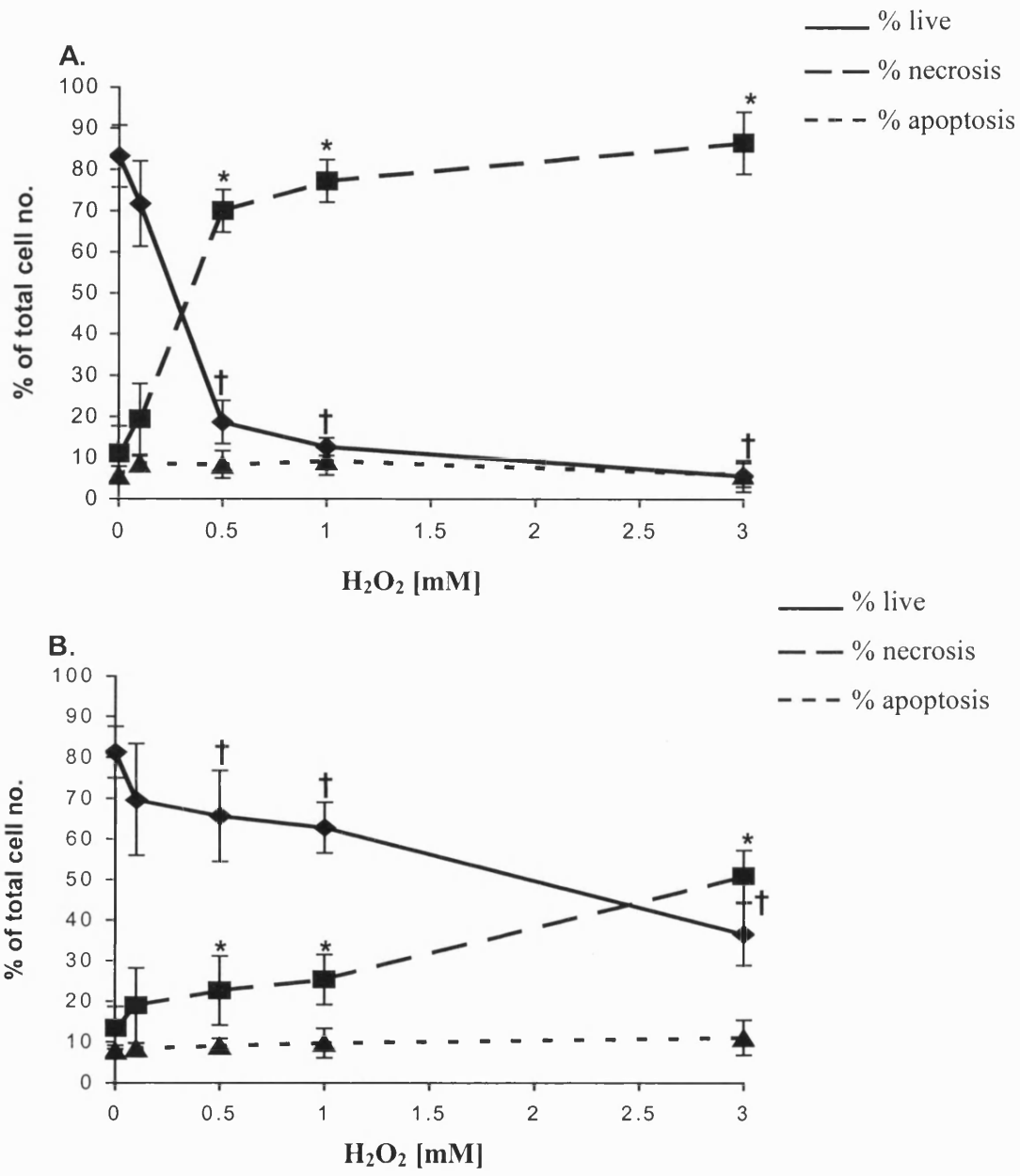



***Figure 3.2:*** Effect of different $H_2O_2$ doses on the survival of parental (A-24 h) and $H_2O_2$ resistant (B-24 h), Jurkat cells.

Note: Cells were incubated for 24 h post-treatment prior to dual Annexin V / PI staining and flow cytometric analysis. Mean ± SD (n = 3)

† : $p<0.05$, significant difference between parental and $H_2O_2$ resistant cells at corresponding $H_2O_2$ doses (live cells)

* : $p< 0.05$, significant difference between parental and $H_2O_2$ resistant cells at corresponding $H_2O_2$ doses (necrotic cells)

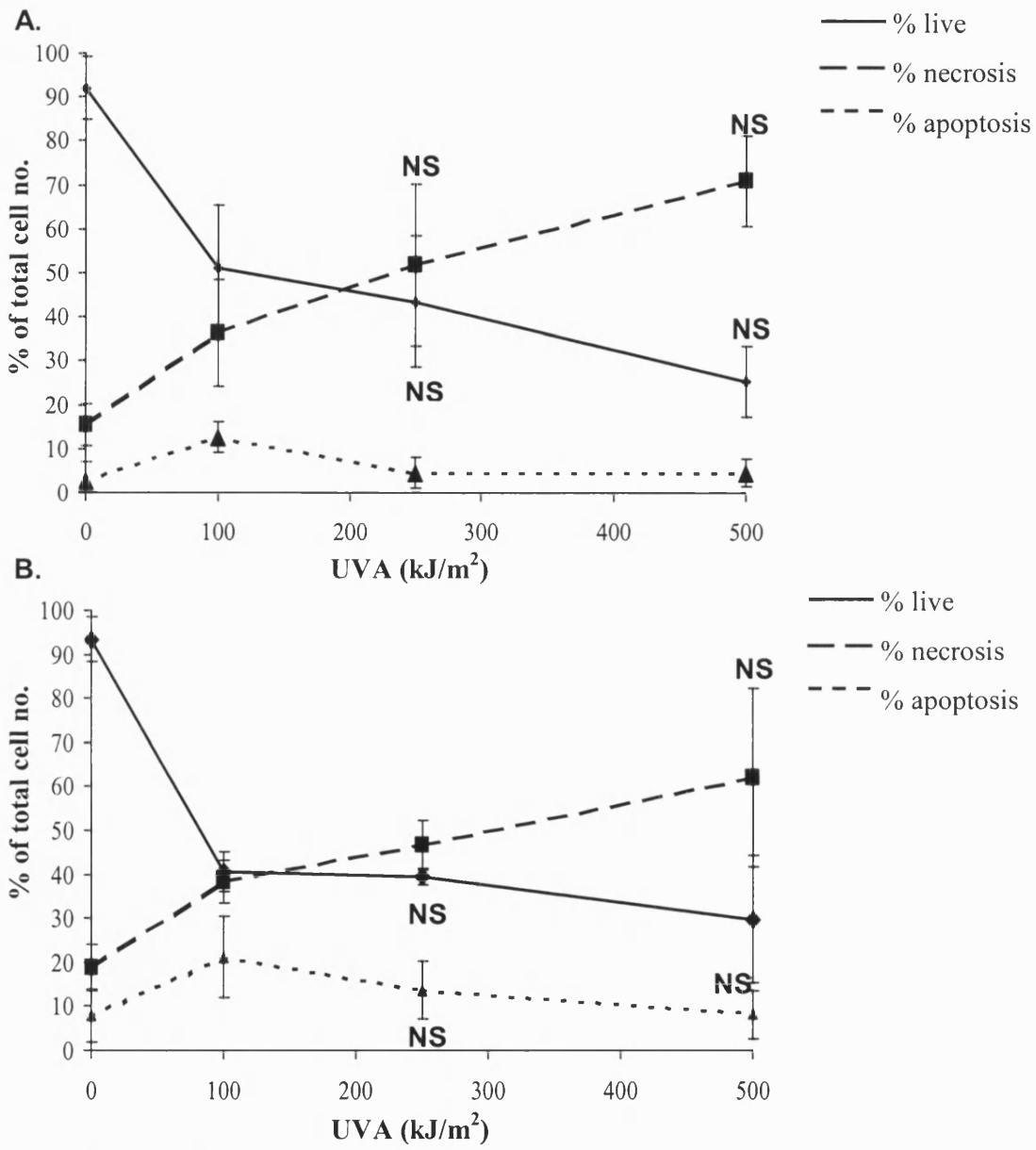



***Figure 3.3:*** Effect of UVA ($kJ/m^2$) on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells.
Note: Cells were incubated for 4 h post-irradiation prior to dual Annexin V / PI staining and flow cytometric analysis.
Mean ± SD (n = 3-4)
NS : $p > 0.05$, no significant difference between parental and UVA resistant cells at corresponded UVA doses

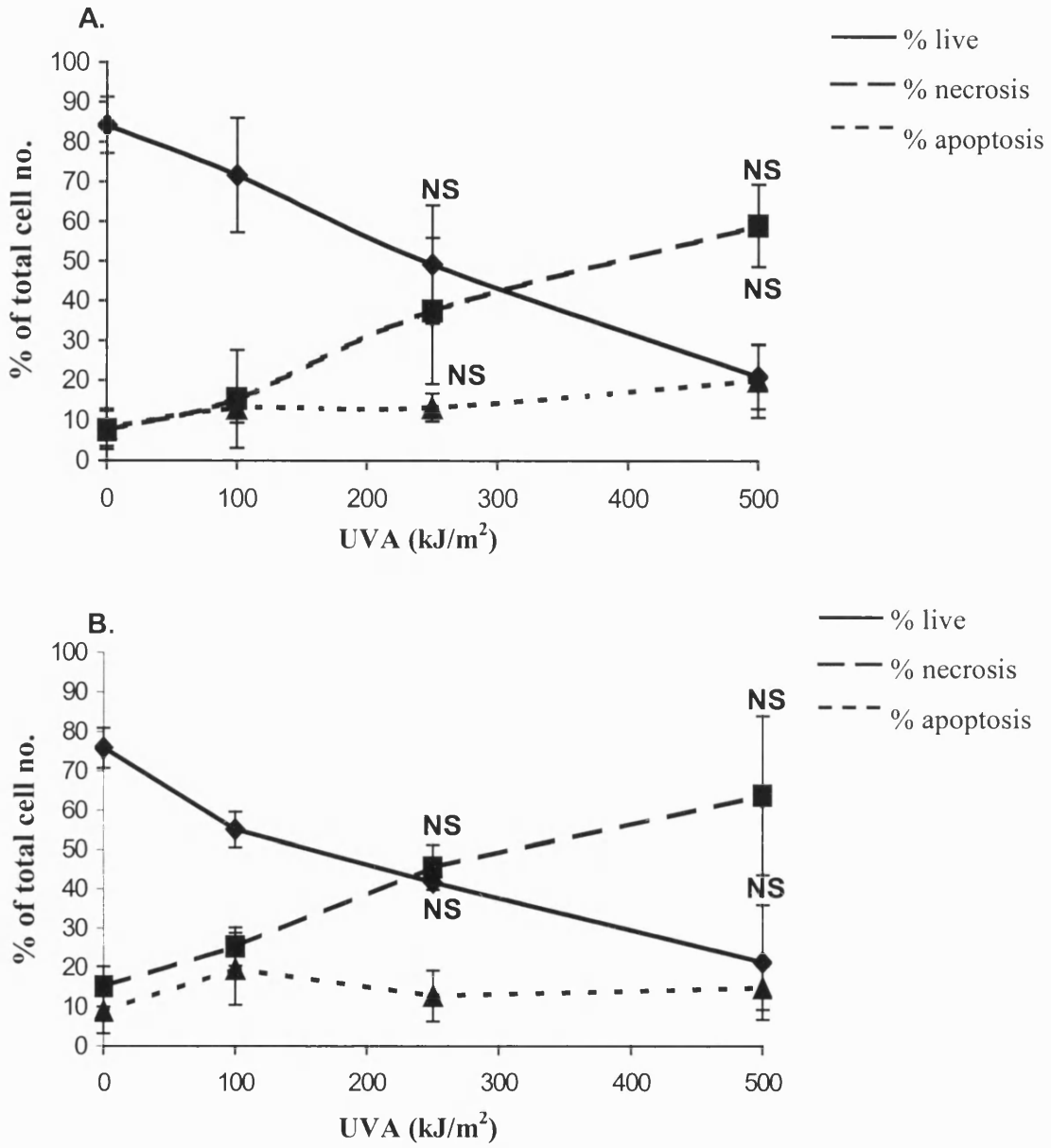



***Figure 3.4:*** Effect of UVA (kJ/m$^2$) on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells.
Note: Cells were incubated for 24 h post-irradiation prior to dual Annexin V / PI staining and flow cytometric analysis.
Mean ± SD (n = 3-4)
NS : $p > 0.05$, no significant difference between parental and UVA resistant cells at corresponded UVA doses

observed either 4 or 24 h following radiation treatment. Furthermore, at both time points, the percentages of apoptotic cells were very low. Overall, these data indicate that Jurkat cells resistant to $H_2O_2$ are not resistant to UVA radiation. Moreover, these data strongly suggest that the primary mode of cell death after exposure of cells to UVA or $H_2O_2$ is necrosis.

### 3.2 The role of intracellular ATP concentration on the switch between apoptosis and necrosis.

Progression of death stimuli to necrosis and apoptosis depends on the effect the mitochondrial damage has on cellular ATP levels (see Reed, 1998; Leist *et al*, 1997; Ha and Snyder, 1999). Apoptosis is an energy driven process and ATP is essential to the early events of apoptosis. In contrast ATP depletion triggers necrosis (see section 1.8). To assess the correlation between the percentage of $H_2O_2$-mediated necrosis in cells (i.e. measured by flow cytometry) and the extent of intracellular ATP depletion, Apoglow kit (see section 2.6) was used to monitor the modulation of the intracellular level of ATP following treatment of both cell lines with $H_2O_2$.

The results in **Figure 3.5** revealed that $H_2O_2$ promotes immediate depletion of ATP in both cell lines in a dose dependent manner as measured within the first 5 min following oxidative insult. Furthermore, ATP depletion does not follow an increase in ADP concentration neither at 10 or 15 min after the addition of the ADP converting reagent indicating that the mode of cell death is predominantly via necrosis. Although, ATP depletion occurred in both cell lines after a range of $H_2O_2$ doses, it appears that the decrease in ATP levels in $H_2O_2$ resistant cells occurs to a lesser extent than in parental cells consistent with the notion that $H_2O_2$ resistant cells are more resistant to

necrosis than parental cells at both 4 and 24 h time points (**Figure 3.6**). These data are also entirely in agreement with the observation made with flow cytometry.

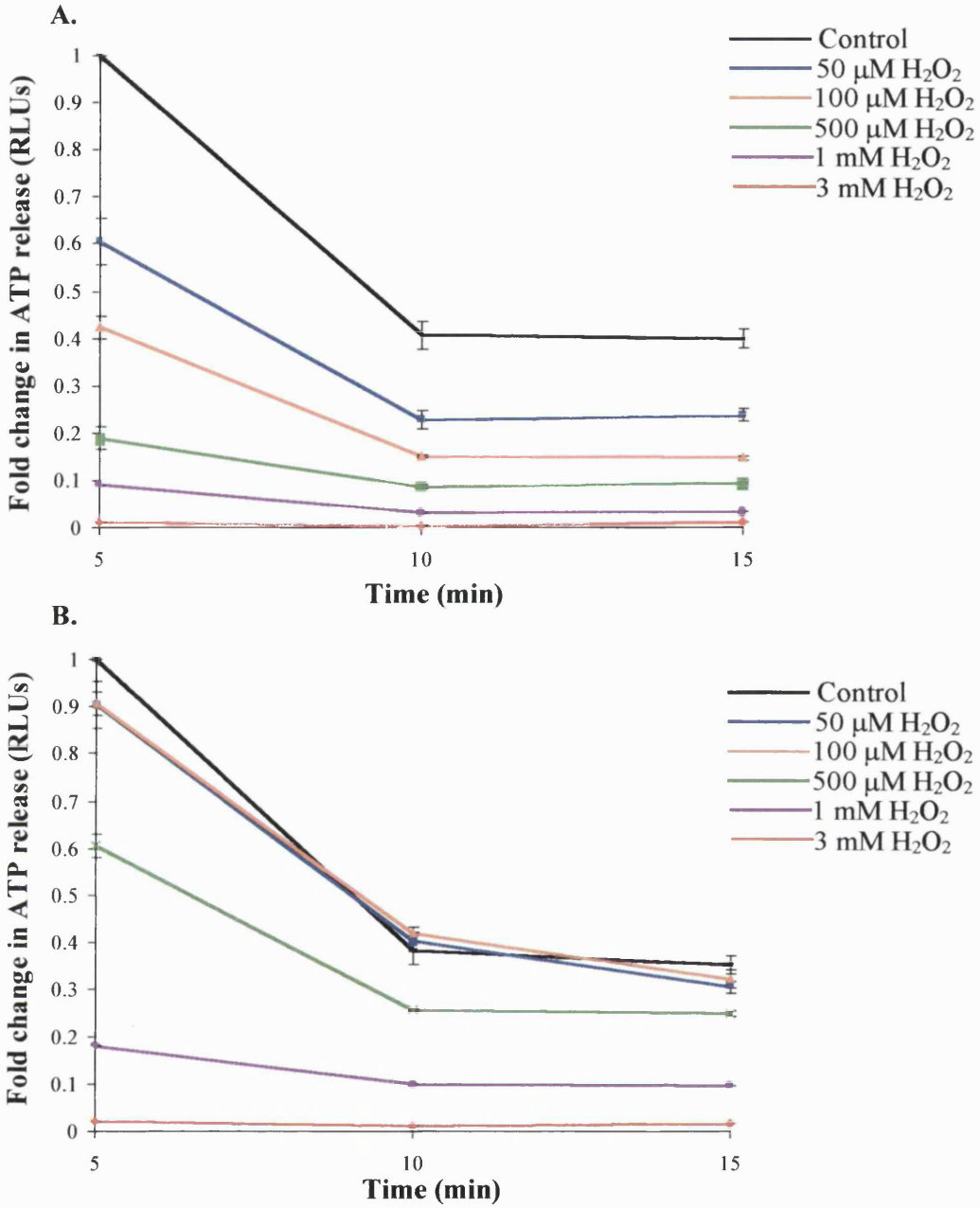



***Figure 3.5*:** A representation of the fold change in ATP release at 4 h after treatment of both parental (A) and $H_2O_2$ resistant (B) cells with a range of doses of $H_2O_2$ using the ApoGlow kit as detailed in section 2.6.
Mean ± SD (n = 3)

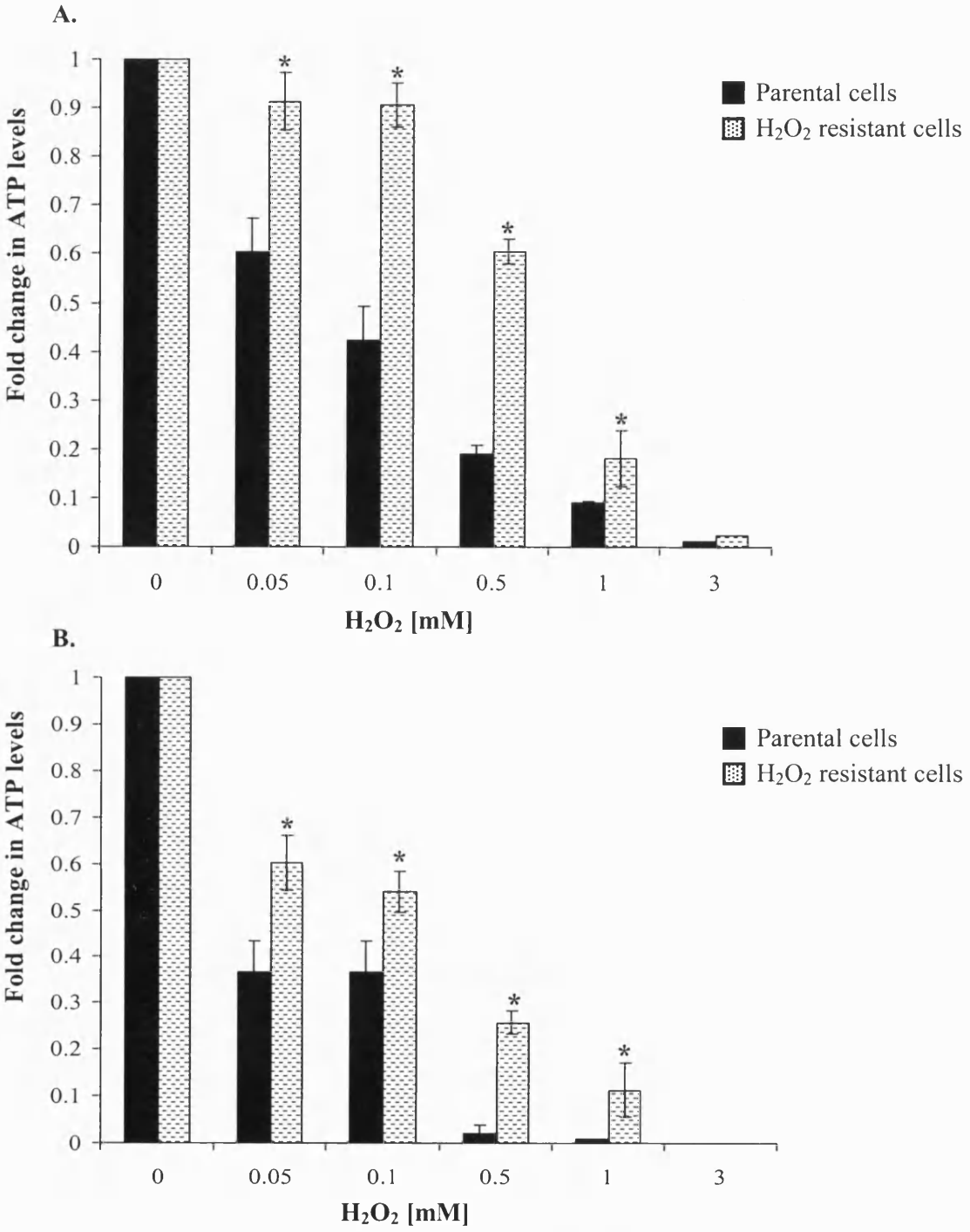



***Figure 3.6*:** Fold change in ATP levels at 4 h (A) and 24 h (B) after a range of doses of $H_2O_2$ treatment in both parental and $H_2O_2$ resistant cells.
Mean ± SD (n = 3)
* : $p<0.05$, significant difference between parental and $H_2O_2$ resistant cells at both 4 and 24h time points.

### 3.3 The role of anti-oxidant enzymes

It is well established that most of the $H_2O_2$ in cells is eliminated by intracellular antioxidant enzymes catalase and GPx. In addition, GSH is present in high concentrations in many cell types and is expected to be important in cellular defence. Thus the role of these anti-oxidant enzymes in resistance of cells to $H_2O_2$ was investigated.

#### *3.3.1* The role of catalase activity

To investigate whether higher catalase activity in cells is related to the higher resistance of cells to $H_2O_2$, the basal level of catalase activity was determined spectrophotometrically by following the rate of $H_2O_2$ consumption as detailed in section 2.11. The results (**Table 3.1**) showed no significant difference in the level of catalase activity between both parental and $H_2O_2$ resistant cells, suggesting that there is no correlation between this enzymatic activity and the resistance of cells to $H_2O_2$.

#### *3.3.2* The role of glutathione peroxidase (GPx)

In addition to catalase, the level of activity of GPx, another $H_2O_2$-detoxyfying enzyme, was measured (see section **2.12**), in both parental and $H_2O_2$ resistant cells. The results (**Table 3.2**) showed that the activity of GPx is significantly different between the parental and $H_2O_2$ resistant cells (*, $p<0.05$). Indeed the GPx activity in $H_2O_2$ resistant cells (124.69 ± 24.59) was up to 1.5 fold higher as compared to the parental cells (88.25 ± 14.67).

Taken together these data indicate that higher GPx activity in $H_2O_2$ resistant cells might play a role in the resistance of cells to $H_2O_2$.

*Table 3.1:* Assessment of catalase activity (u / mg protein) based on $H_2O_2$ consumption, in both parental and $H_2O_2$ resistant Jurkat cells.

| Cell LIne | Catalase activity (u per mg protein) | Fold difference |
|---|---|---|
| ***Parental cells*** | 0.35 ± 0.12 | 1 |
| ***$H_2O_2$ resistant cells*** | 0.29 ± 0.11 $^{NS}$ | 1.2 ± 0.02 |

Note: Catalase activity was measured immediately after lysis of the cells as described in section 2.12.
Mean ± SD (n = 4)
NS : $p > 0.05$, no significant difference between parental and $H_2O_2$ resistant cells

*Table 3.2:* Assessment of GPx activity based on NADPH consumption rate (u / mg protein) in both parental and $H_2O_2$ resistant Jurkat cells.

| Cell line | GPx activity (u per mg protein) | Fold difference |
|---|---|---|
| ***Parental cells*** | 88.25 ± 14.67 | 1 |
| ***$H_2O_2$ resistant cells*** | 124.69 ± 24.59 * | 1.42 ± 0.21 |

Note: GPx activity was measured immediately after treatment of the cells as described in section 2.13.
Mean ± SD (n = 5)
* : $p < 0.05$, significant difference between normal and $H_2O_2$ resistant cells

*3.3.3* The role of glutathione (GSH)

Previous evidences from this laboratory have suggested that GSH plays a critical role as an anti-oxidant compound as depletion of intracellular GSH with BSO was found to sensitise cells to the lethal action of both UVA and UVB radiations as well as $H_2O_2$ (Tyrrell and Pidoux, 1986 and 1988, and unpublished data, this laboratory).

In this study, GSH levels were assessed with or without treatment of both parental and $H_2O_2$ resistant cells with 50 μM BSO for 18 h. The results (**Table 3.3**) showed that total GSH levels are 1.6 fold higher in $H_2O_2$ resistant cells when compared to the parental cells. Furthermore the 50 μM BSO completely abolished the level of GSH in both parental and $H_2O_2$ resistant cells. These data strongly suggest that higher GSH in $H_2O_2$ resistant cells might play a role in their resistance to $H_2O_2$.

*Table 3.3:* Assessment of GSH levels (μM) per mg total protein in both parental and $H_2O_2$ resistant Jurkat cells.

| Total μM GSH per mg protein | | | |
|---|---|---|---|
| **Cell Line** | **Control** | **Fold difference** | **50 μM BSO$^{18h}$** |
| ***Parental cells*** | 0.156 ± 0.041 | 1 | 0.004 ± 0.0008 |
| ***$H_2O_2$ resistant cells*** | 0.25 ± 0.072 * | 1.6 ± 0.05 | 0.002 ± 0.00006 |

Note: GSH levels were measured in untreated control and 50 μM BSO treated cells. The assay was carried out immediately after lysis of the cells and normalised using a standard curve of GSSG, as described in section 2.14.
Mean ± SD (n = 4)
* : $p < 0.05$, significant difference between parental and $H_2O_2$ resistant cells at the control levels

**3.4 Effect on modulation of LIP levels in response to oxidant- induced damage.**

Recent discoveries from our laboratory (Pourzand *et al*, 1999 and unpublished data, this laboratory) indicated that both UVA and $H_2O_2$ as oxidizing agents trigger the immediate increase in LIP within cultured human skin cells. The increase in LIP has also recently been linked in our laboratory to the increased susceptibility of cells to necrotic cell death (unpublished data, this laboratory). These observations prompted us to characterise this phenomenon in our model of human Jurkat T cells and to ask the question as to whether both 'basal'or 'oxidant-induced' increase in LIP play a role in resistance of Jurkat cells to UVA and $H_2O_2$ – mediated necrotic cell death. **Table 3.4** summarises the basal level concentration of LIP in both cell lines of parental and $H_2O_2$ resistant, as determined by the Kd values with CA-assay for each cell line (see section 2.7). The results demonstrated that the basal level of LIP were similar in both cell lines. The determination of Kd values and the cell volume were required for calibration of LIP concentration. The Kd values were not significantly different between parental (28.82 ± 5.06) and $H_2O_2$ resistant (20.06 ± 2.51) cells. In contrast, the cell volume of $H_2O_2$ resistant cells (1.6 ± 0.19) was 2 fold higher than that of parental cells (0.85 ± 0.06).

*3.4.1* The role of LIP on the increased susceptibility of cells to $H_2O_2$ resistance

Using the CA-assay (see the section 2.7), the LIP levels were measured immediately after $H_2O_2$ treatment of both parental and $H_2O_2$ resistant cells with $H_2O_2$ doses of 0.05 to 3 mM. The results (**Figure 3.7**) revealed that following $H_2O_2$ treatment with non-lethal doses of $H_2O_2$ (i.e. 0.05 – 1 mM), the level of LIP in both cell lines was increased in a dose-dependent manner. The comparison of $H_2O_2$-

mediated LIP release in both parental and $H_2O_2$ resistant cell lines revealed that the extent of LIP release in parental cells was up to 3.5 fold higher than the $H_2O_2$ resistant cells. The latter difference in LIP release was more significant at the first three doses of $H_2O_2$ (i.e. 50, 100 and 500 μM) treatment (*, $p<0.05$). Interestingly, when cells were exposed to 1 and 3 mM doses of $H_2O_2$, the LIP levels began to decrease, presumably due to higher toxicity of the doses applied (see **Figure 3.2**). These results strongly suggested a direct correlation between the level of $H_2O_2$-induced labile iron release and the level of resistance of cells to $H_2O_2$.

*3.4.2* The role of LIP in response to UVA

To study the effect of UVA on modulation of LIP levels in both parental and $H_2O_2$ resistant cells, cells were irradiated with UVA doses of 250 and 500 $kJ/m^2$ and the change in LIP levels were then assessed by the CA-assay. The results (**Figure 3.8**) revealed that UVA triggers labile iron release in both cell lines in a dose-dependent manner. Indeed at a high dose of UVA (500 $kJ/m^2$), the LIP levels were significantly higher in both treated cell lines when compared to the corresponding untreated control cells. However at a moderate dose of 250 $kJ/m^2$, a significant increase in LIP was observed in $H_2O_2$ resistant cells when compared to the parental cells. Indeed following a UVA dose of 250 $kJ/m^2$, labile iron release in $H_2O_2$ resistant cells was up to 2 fold higher than the parental cells (*, $p<0.05$). These data indicate that although UVA radiation induces labile iron release in both cell lines, the extent of this phenomenon is much higher in $H_2O_2$ resistant cell line when compared to parental cells. So, these results clearly indicated that the effects of $H_2O_2$ and UVA on the level of LIP release are opposite in both parental and $H_2O_2$ resistant cells.

*Table 3.4*: Determination of basal LIP in parental and $H_2O_2$ resistant Jurkat cells.

| Cell line | Cell Volume | Kd value | [LIP] μM |
|---|---|---|---|
| ***Parental cells*** | 0.85 ± 0.006 | 28.82 ± 5.06 | 3.08 ± 0.59 |
| ***$H_2O_2$ resistant cells*** | 1.6 ± 0.19 | 20.06 ± 2.51 | 3.34 ± 0.87 |

Note: The LIP concentrations are expressed as mean ± SD (n=3-16). The cell volume and Kd values are also expressed as mean ± SD (n=3-6).

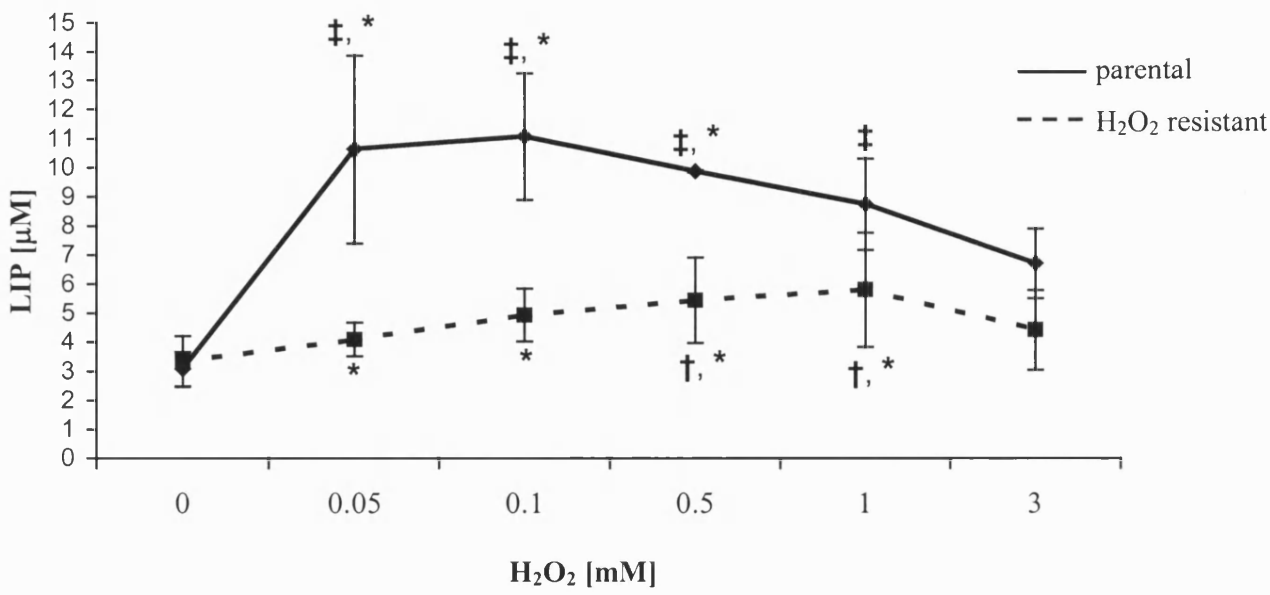



***Figure 3.7:*** Effect of $H_2O_2$ treatment on the concentration of LIP in both parental and $H_2O_2$ resistant Jurkat cells. LIP determination was performed immediately after $H_2O_2$ treatment.
Mean ± SD (n =3-8)
* : $p<0.05$, significant difference between parental and $H_2O_2$ resistant cells, at corresponding $H_2O_2$ doses.
‡, † : $p<0.05$, significantly different from untreated control.

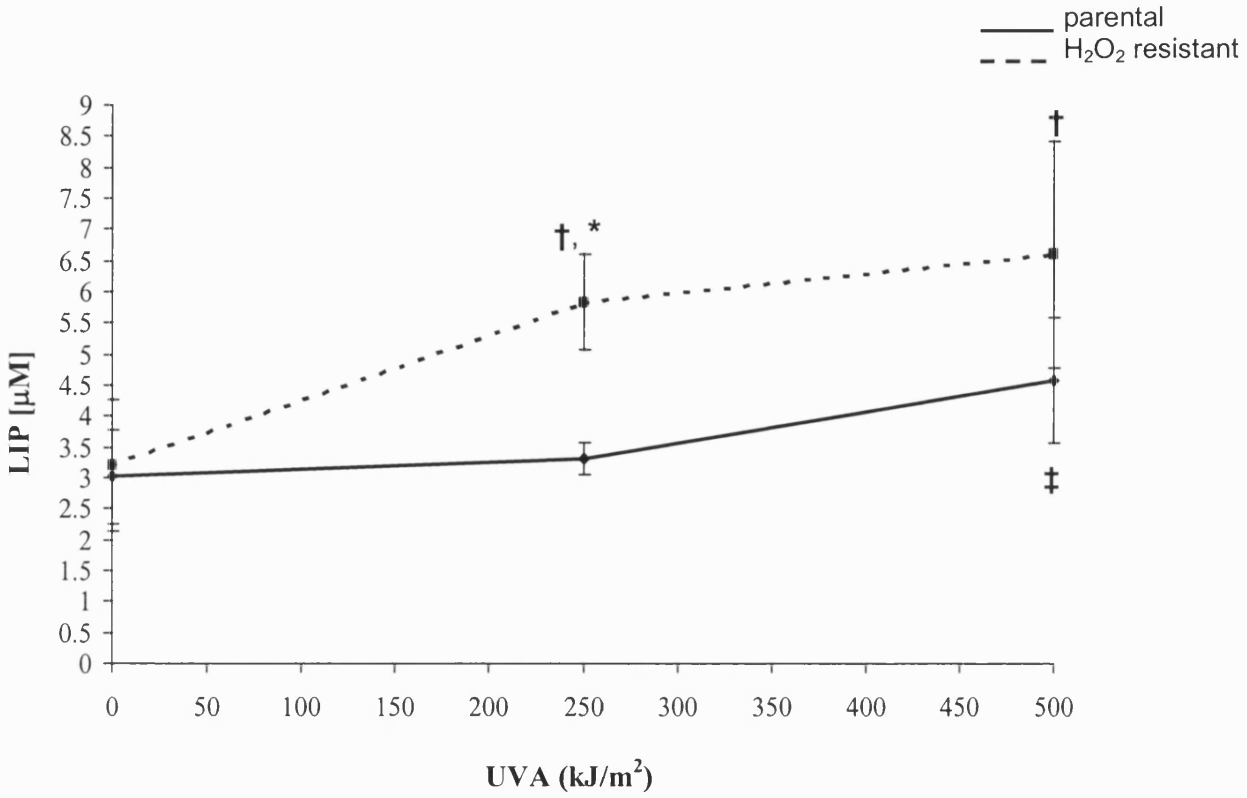



***Figure 3.8:*** Effect of UVA irradiation on the concentration of LIP in parental and $H_2O_2$ resistant Jurkat cells. Measurements were performed immediately after irradiation.
Mean ± SD (n = 3)
* : $p<0.05$, significant difference comparing parental and $H_2O_2$ resistant cells at the corresponding UVA dose.
† : $p<0.05$, significant different compared to untreated control in $H_2O_2$ resistant cells.
‡ : $p<0.05$, significant different compared to untreated control in parental cells.

## 3.5 The role of intracellular LIP level in modulating the resistance of cells to $H_2O_2$.

To assess the hypothesis that changes in the intracellular level of labile iron might in turn modulate the resistance of cells to $H_2O_2$, cells were first incubated with either 100 μM DFO (iron chelation) or 20 μM hemin (iron loading) prior to treatment with $H_2O_2$. Next, the change in LIP was monitored by the CA assay and the level of resistance of cells to $H_2O_2$ was assessed by both MTT and Annexin V/PI staining assays. The results are discussed below in sections **3.5.1** to **3.5.4**.

### *3.5.1* Effects of iron depletion on the cell survival

Parental and $H_2O_2$ resistant cells were first incubated for 18 h with 100 μM DFO and then treated with a range of doses of $H_2O_2$. The assessment of cell survival was performed by MTT assay 4 and 24 h following $H_2O_2$ treatment. The results revealed that in parental cells at both 4 (**Figure 3.9**) and 24 h (**Figure 3.10**) post-treatment time points, the percentage of live cells in $H_2O_2$ + DFO treated cells was significantly higher (*, $p<0.05$) than in $H_2O_2$ treated cells alone. On the other hand, in $H_2O_2$ resistant cells the protective effect of DFO was only apparent at a high dose of 3 mM.

We also evaluated the effect of DFO on the cell survival following UVA radiation of both parental and $H_2O_2$ resistant cell lines. For this purpose cells were first

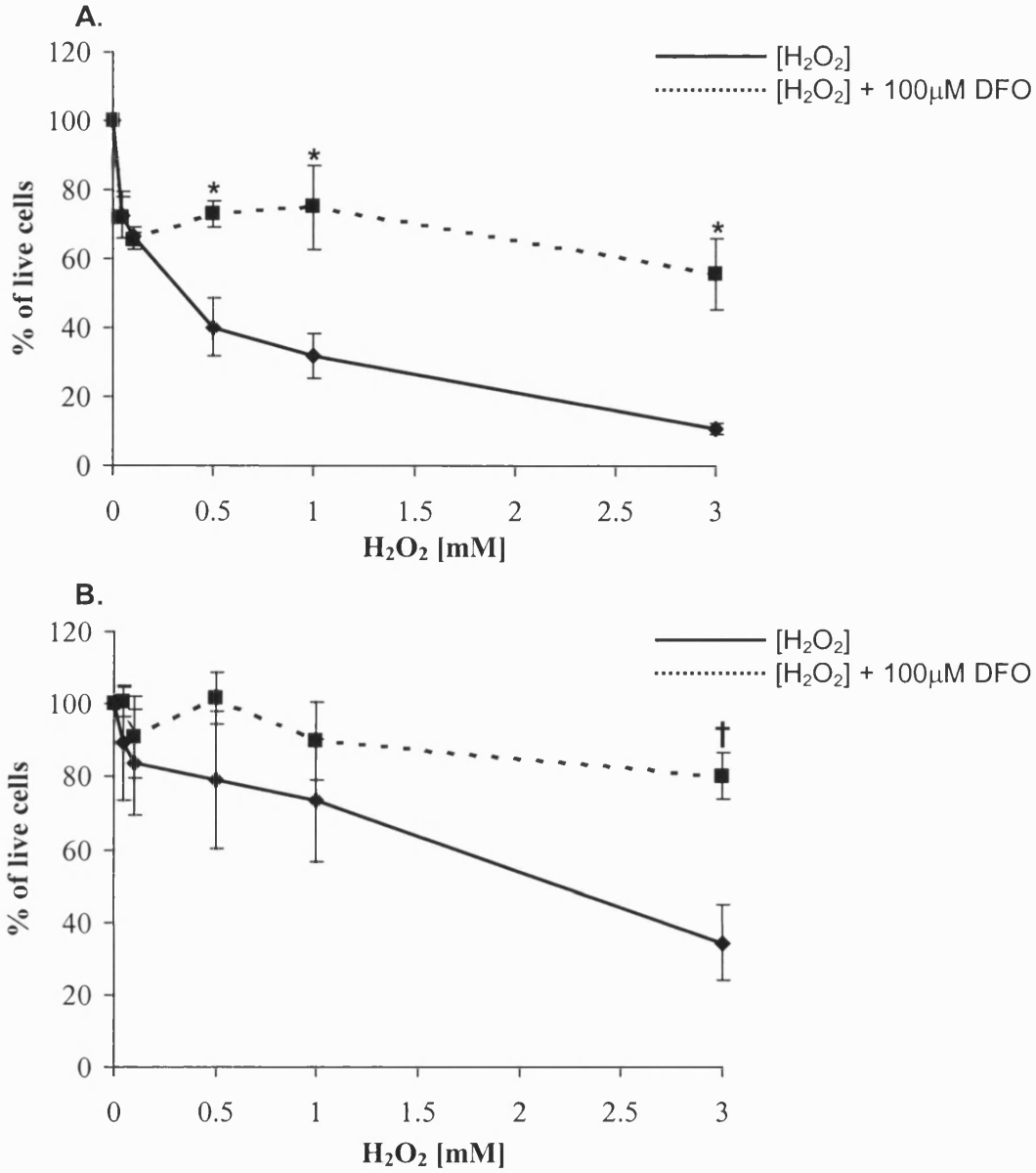



***Figure 3.9***: Effect $H_2O_2$ ± DFO treatment on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells. Both cell lines were treated with 100 μM DFO for 18 h before $H_2O_2$ treatment. In both cell lines MTT assay was performed 4h following $H_2O_2$ treatment.
Mean ± SD (n=3)
* : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + DFO treated, in parental cells
† : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + DFO treated, in $H_2O_2$ resistant cells

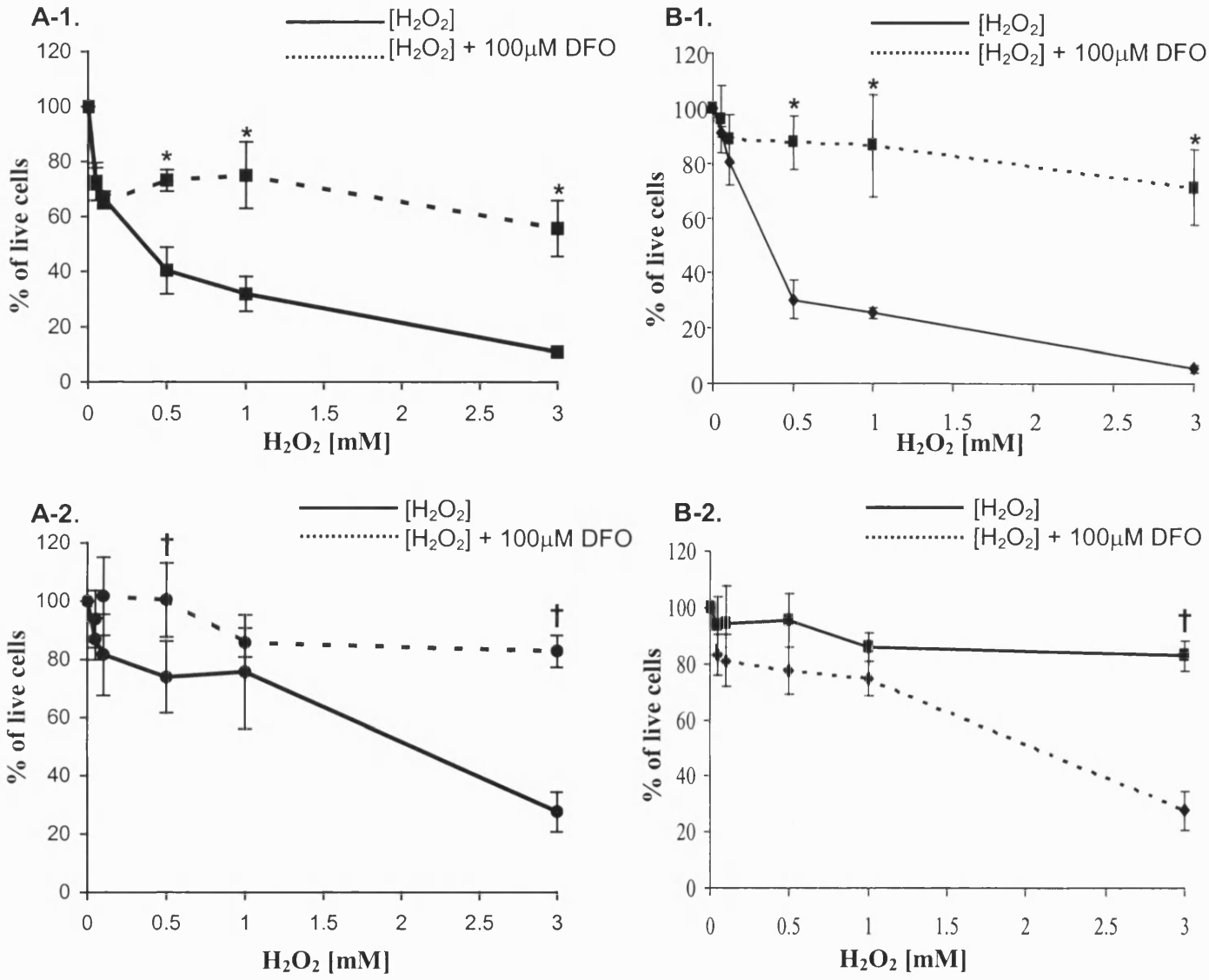



***Figure 3.10***: Effect $H_2O_2$ ± DFO treatment on the survival of parental (A-1 and B-1) and $H_2O_2$ resistant (A-2 and B-2), Jurkat cells. Both cell lines were treated with 100 μM DFO for 18 h before $H_2O_2$ treatment. In both cell lines MTT assay (A-1 and A-2) and flow cytometric analysis (B-1 and B-2) were performed 24 h following $H_2O_2$ treatment.

Mean ± SD (n=3)

* : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + DFO treated, in parental cells

† : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + DFO treated, in $H_2O_2$ resistant cells

incubated for 18h with 100 μM DFO and then irradiated with UVA doses of 100, 250 and 500 kJ/m$^2$. The MTT assay was performed 4 and 24 h following UVA radiation treatment. The results (**Figure 3.11**) demonstrated that DFO treatment *per se* does not affect the percentage of cell survival 4 h after UVA radiation in both cell lines. However, at 24 h post-treatment time point, the percentage of live cells dropped dramatically in both UVA treated parental and $H_2O_2$ resistant cells (**Figure 3.12**) indicating that cells resistant to $H_2O_2$ are not necessarily resistant to UVA radiation. Since the percentage of live cells was generally higher in DFO-treated cells, it was concluded that pre-treatment with DFO protects the parental and $H_2O_2$ resistant cells against UVA radiation.

*3.5.2* Effects of iron depletion on the levels of LIP release

Treatment of parental and $H_2O_2$ resistant cells with 100 μM DFO for 18 h completely abolished both the basal and the $H_2O_2$-induced levels of LIP (**Table 3.5**). These results were in agreement with findings by Pourzand *et al* (1999) showing that treatment of a human primary fibroblast cell line with DFO prior to UVA, can abolish both the basal and UVA-induced level of LIP.

*3.5.3* Effects of iron loading on the cell survival

Parental and $H_2O_2$ resistant cells were first incubated for 18 h with 20 μM hemin prior to $H_2O_2$ treatment. The MTT assay was then performed (see section 2.5) 4 and 24 h following $H_2O_2$ treatment. In addition to MTT assay, quantification of apoptosis and necrosis was also performed by the dual Annexin V/PI staining assay (see section 2.4) 24 h following exposure of both cell lines to $H_2O_2$. The MTT assay (**Figure 3.13**) performed 4 h following $H_2O_2$ treatment revealed that at high doses of 1 and 3 mM

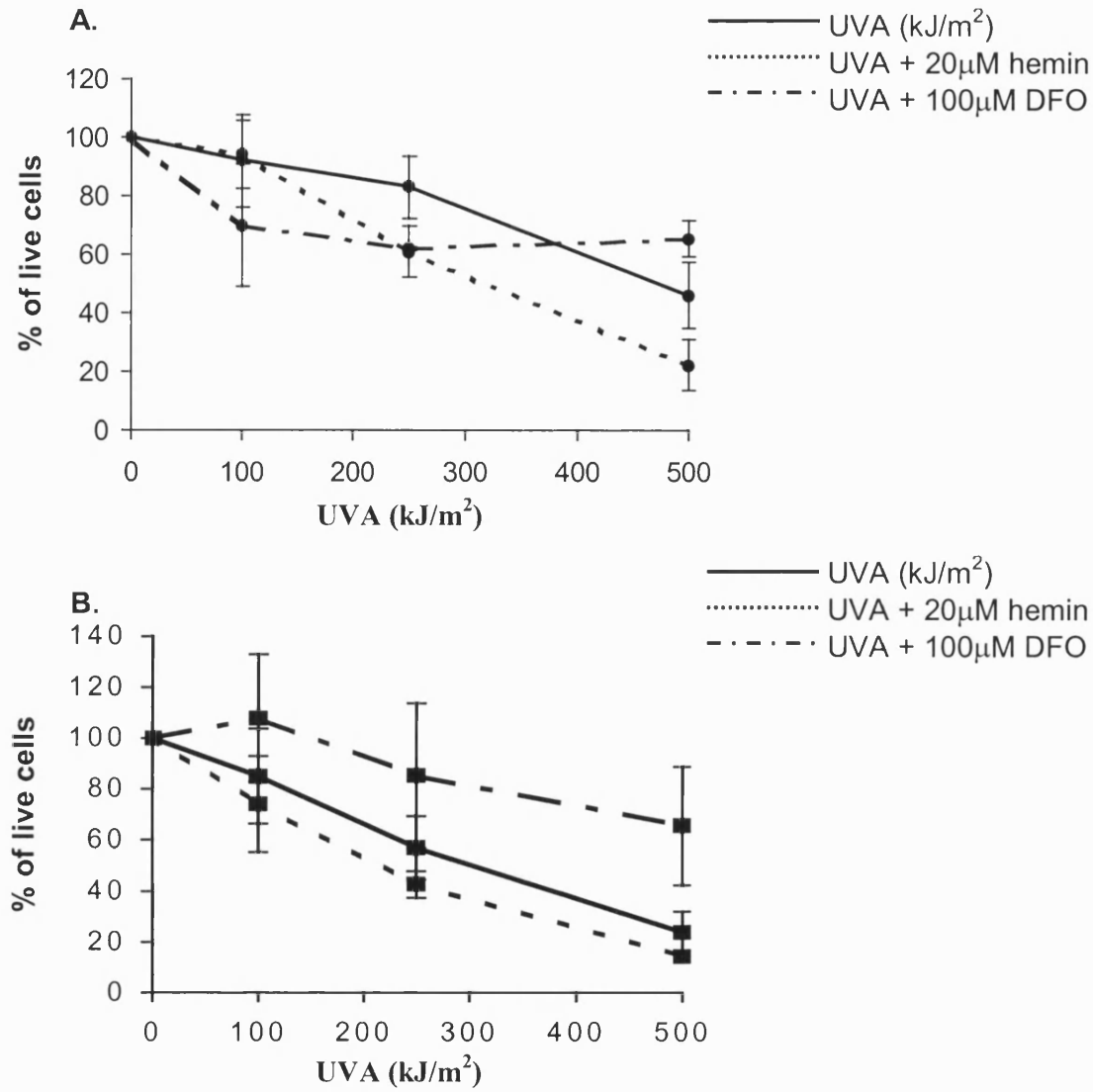



***Figure 3.11***: Effect of UVA ± hemin ± DFO treatments on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells. Both cell lines were treated either with 20 μM hemin or with 100 μM DFO, for 18 h before UVA radiation. In both cell lines MTT assay was performed 4 h following UVA radiation.
Mean ± SD (n=3)
According to statistical analysis by *t*-test no significant difference was observed between UVA-treated and UVA + hemin or DFO treated, cells.

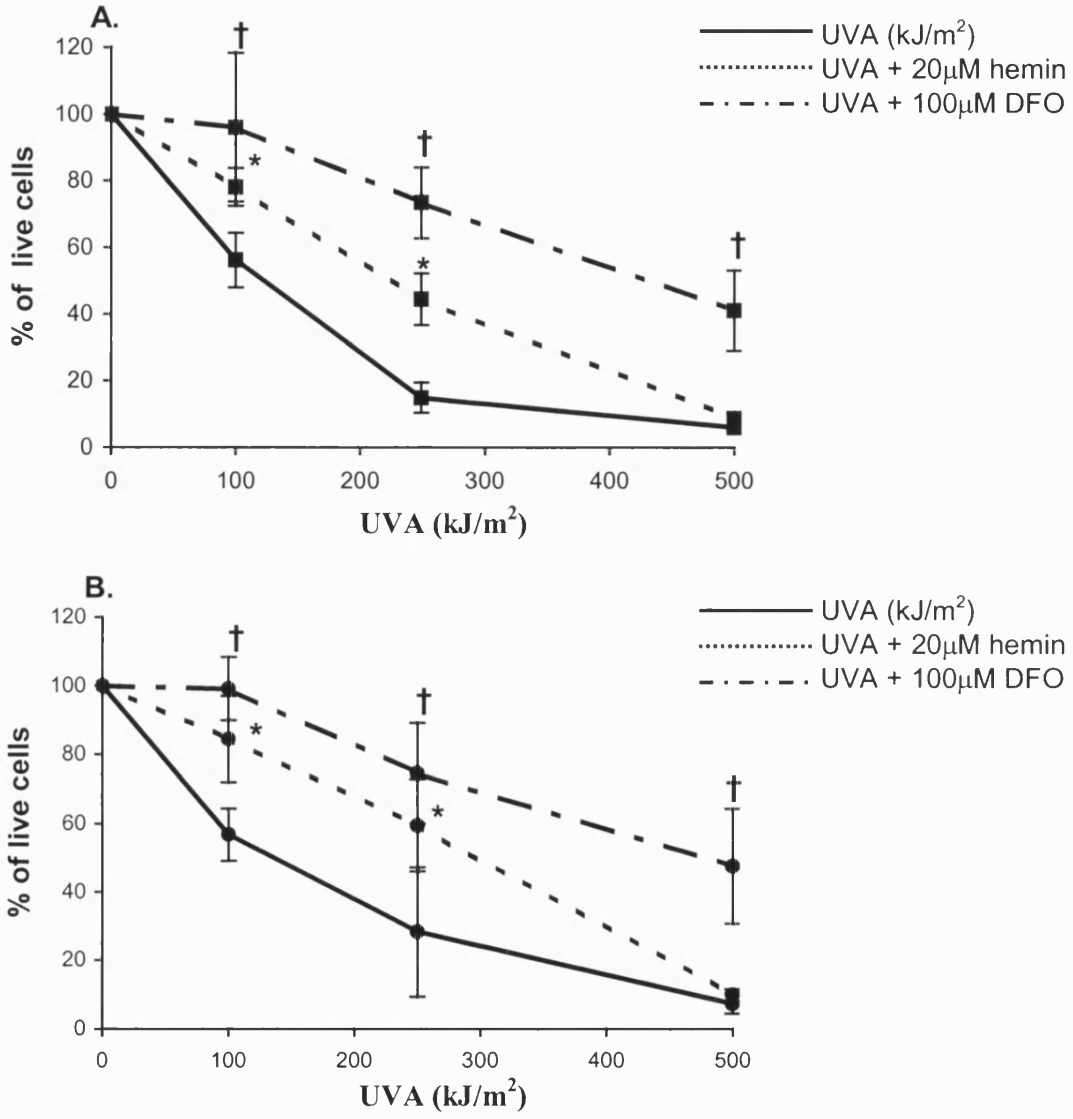



***Figure 3.12*:** Effect of UVA ± hemin ± DFO treatments on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells. Both cell lines were treated either with 20 μM hemin or with 100 μM DFO, for 18 h before UVA radiation. In both cell lines MTT assay was performed 24 h after UVA radiation.
Mean ± SD (n=3)
* : $p < 0.05$, significant difference between UVA treated and $H_2O_2$ + hemin treated (in both cell lines)
† : $p < 0.05$, significant difference between UVA treated and $H_2O_2$ + DFO treated (in both cell lines)

*Table 3.5*: Determination of basal level of LIP in parental and $H_2O_2$ resistant Jurkat cells.

| Cell line | Cell Volume | Kd value | [LIP] μM | [$LIP^H$] μM | [$LIP^{DFO}$] μM |
|---|---|---|---|---|---|
| ***Parental cells*** | 0.85 ± 0.006 | 28.82 ± 5.06 | 3.08 ± 0.59 | 2.93 ± 0.73 | 0 ± 0 |
| ***$H_2O_2$ resistant cells*** | 1.6 ± 0.19 | 20.06 ± 2.51 | 3.34 ± 0.87 | 7.2 ± 0.56 | 0 ± 0 |

Note: The LIP concentrations are expressed as mean ± SD (n=3-16). $LIP^H$ represents the LIP concentrations measured after 18h of 20μM hemin treatment in parental and $H_2O_2$ resistant Jurkat cells. $LIP^{DFO}$ represents the LIP concentrations measured after 18 h of 100 μM DFO treatment in parental and $H_2O_2$ resistant cells. The cell volume and Kd values are also expressed as mean ± SD (n=3-6).

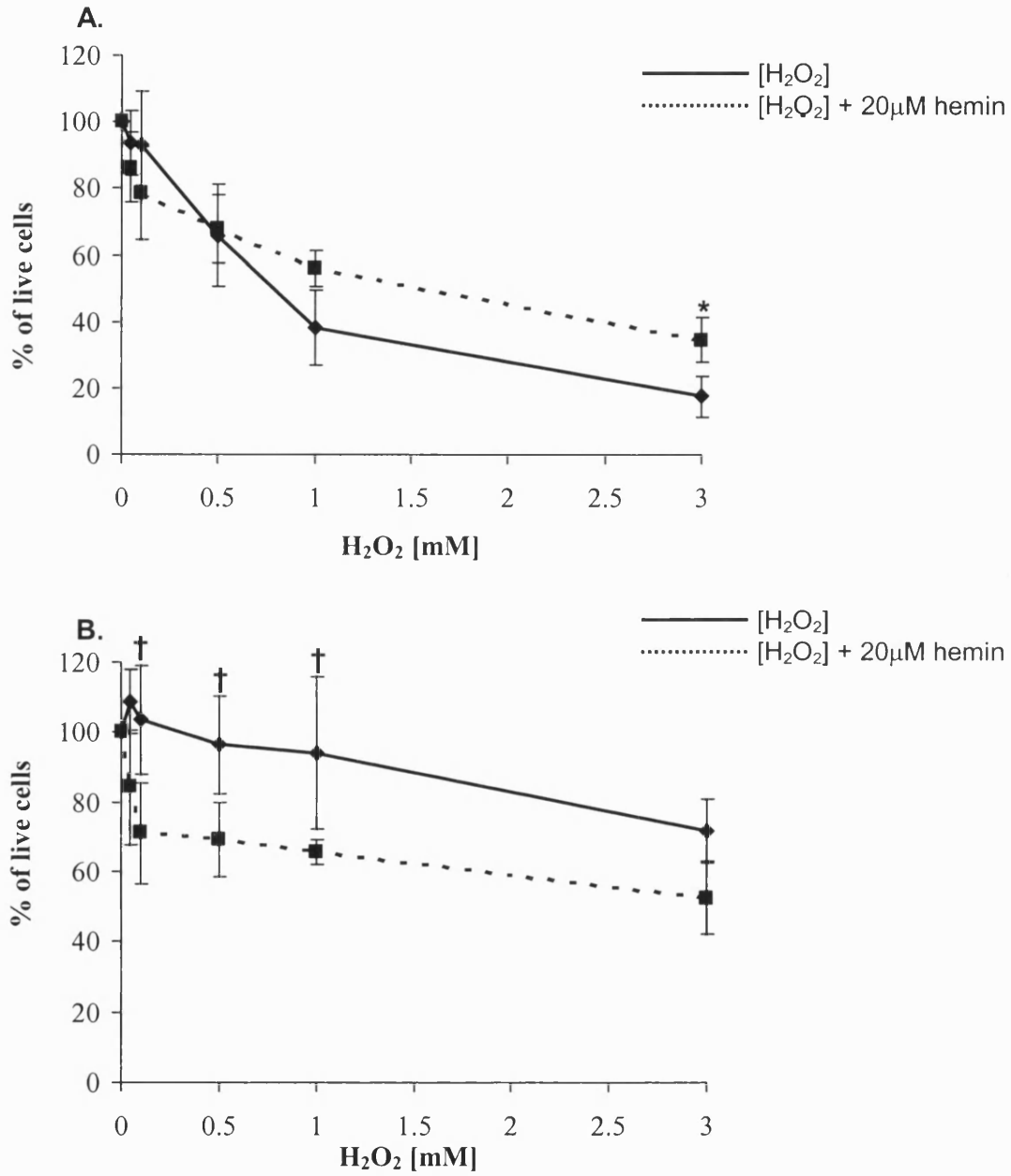



***Figure 3.13:*** Effect of $H_2O_2$ on the survival of parental (A) and $H_2O_2$ resistant (B), Jurkat cells with or without hemin treatment. Both cell lines were treated with 20 μM hemin for 18 h prior to $H_2O_2$ treatment. In both cell lines MTT assay was performed 4 h following $H_2O_2$ treatment.

Mean ± SD (n=3)

* : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + hemin treated, in parental cells

† : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + hemin treated, in $H_2O_2$ resistant cells

$H_2O_2$, the percentage of cell survival in $H_2O_2$ + hemin treated cells was significantly higher than the $H_2O_2$ treated cells alone. In contrast in the $H_2O_2$ resistant cells the effects were exactly the opposite (i.e. the percentage of cell survival in $H_2O_2$ + hemin treated cells were lower than the $H_2O_2$ treated cells alone). At the 24 h post-treatment time points both MTT and flow cytometric analyses showed (**Figure 3.14**) that the percentage of live cells in $H_2O_2$ + hemin treated cells was still higher in parental cells, but not in $H_2O_2$ resistant cells. Taken together, these results indicated that hemin treatment on the one hand protects parental cells against $H_2O_2$ treatment and on the other hand sensitises $H_2O_2$ resistant cells to $H_2O_2$ treatment.

We also evaluated the effect of hemin on cell survival following UVA radiation of both parental and $H_2O_2$ resistant cell lines. For this purpose cells were first incubated for 18 h with 20 μM hemin and then irradiated with UVA doses of 100, 250 and 500 $kJ/m^2$. The MTT and flow cytometric assays were performed 4 and 24 h following UVA irradiation of both cell lines. The analyses performed 4 h following UVA irradiation (**Figure 3.11**) demonstrated that hemin pre-treatment did not affect cell survival in either of the cell lines. However, at 24 h post-treatmet time point, the percentage of live cells dropped dramatically in both UVA treated parental and $H_2O_2$ resistant cells (**Figure 3.12**), consistent with the notion that cells resistant to $H_2O_2$ are not necessarily resistant to UVA radiation. The percentage of live cells was higher in hemin pre-treated cells when compared to UVA irradiated cells alone. So, it appears that hemin pre-treatment protects the parental and $H_2O_2$ resistant cells against UVA radiation.

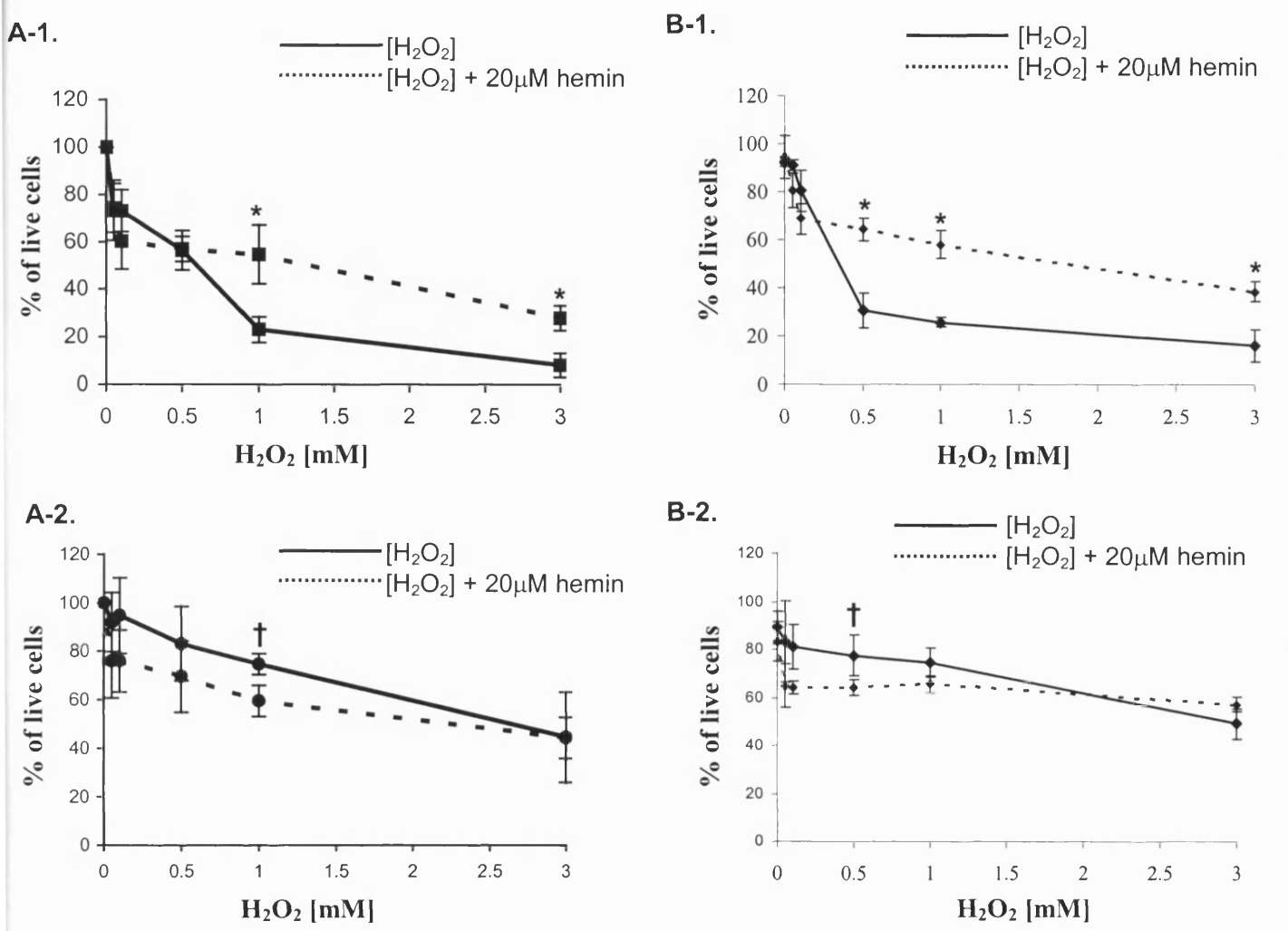



***Figure 3.14:*** Effect of $H_2O_2$ on the survival of parental (A-1 and B-1) and $H_2O_2$ resistant (A-2 and B-2) Jurkat cells with or without hemin treatment. Both cell lines were treated with 20 μM hemin for 18 h prior to $H_2O_2$ treatment. In both cell lines MTT assay (A-1 and A-2) and Flow Cytometry (B-1 and B-2) were performed 24 h following $H_2O_2$ treatment.

Mean ± SD (n=3)

* : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + hemin treated, in parental cells

† : $p < 0.05$, significant difference between $H_2O_2$ treated and $H_2O_2$ + hemin treated, in $H_2O_2$ resistant cells

*3.5.4* Effects of iron loading on $H_2O_2$ mediated LIP release

In order to explain the differential effects of hemin after $H_2O_2$ treatment between parental and $H_2O_2$ resistant cells, the LIP levels were measured immediately after $H_2O_2$ treatment. As shown in **Table 3.5**, the basal LIP levels, after 18 h hemin treatment, were up to 2.5 fold higher in $H_2O_2$ resistant cells (7.2 ± 0.56 μM) as compared to parental cells (2.93 ± 0.73 μM). Following $H_2O_2$ treatment LIP release occurred only in $H_2O_2$ resistant cells, while in parental cells the LIP level was not significantly different from the basal levels (**Figure 3.15**). Indeed, when $H_2O_2$ resistant cells were treated with 50 μM $H_2O_2$, the LIP level increased up to 4 fold when compared to untreated control cells. However at higher doses (i.e. 100 and 500 μM $H_2O_2$) the LIP release occurred to a lesser extent. **Figure 3.16** shows the level of $H_2O_2$-mediated LIP release in each cell line with or without hemin pre-treatment. It appears that hemin pre-treatment decreases dramatically the level of oxidant-induced LIP release in the parental cell line. On the other hand the same pre-treatment boosts up to several fold the level of $H_2O_2$-induced LIP release in $H_2O_2$-resistant cells. The latter results show a direct correlation between the extent of LIP release in both cell lines and the level of resistance of cells to $H_2O_2$.

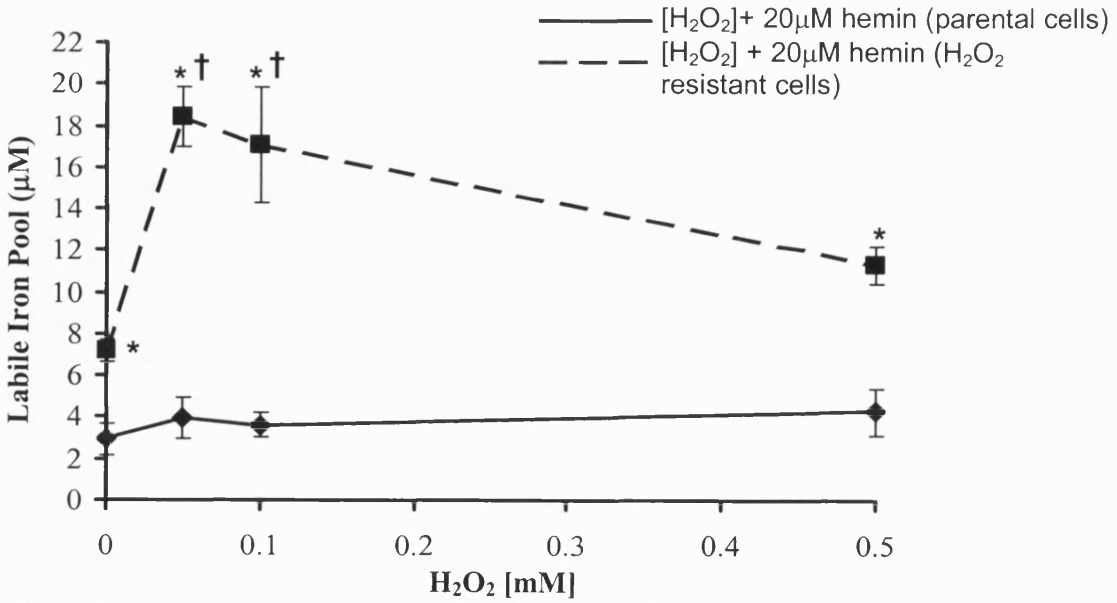



***Figure 3.15*:** Effect of $H_2O_2$ on the concentration of labile iron pool in both parental and $H_2O_2$ resistant Jurkat cells with or without hemin treatment. Both cell lines were treated with 20 μM hemin for 18 h prior to $H_2O_2$ treatment. The Calcein assay was performed immediately after $H_2O_2$ treatment.
Mean ± SD (n =3-6)
* : $p<0.05$, significant difference between normal and $H_2O_2$ resistant cells, at corresponded $H_2O_2$ doses
† : $p<0.05$, significantly different from untreated control

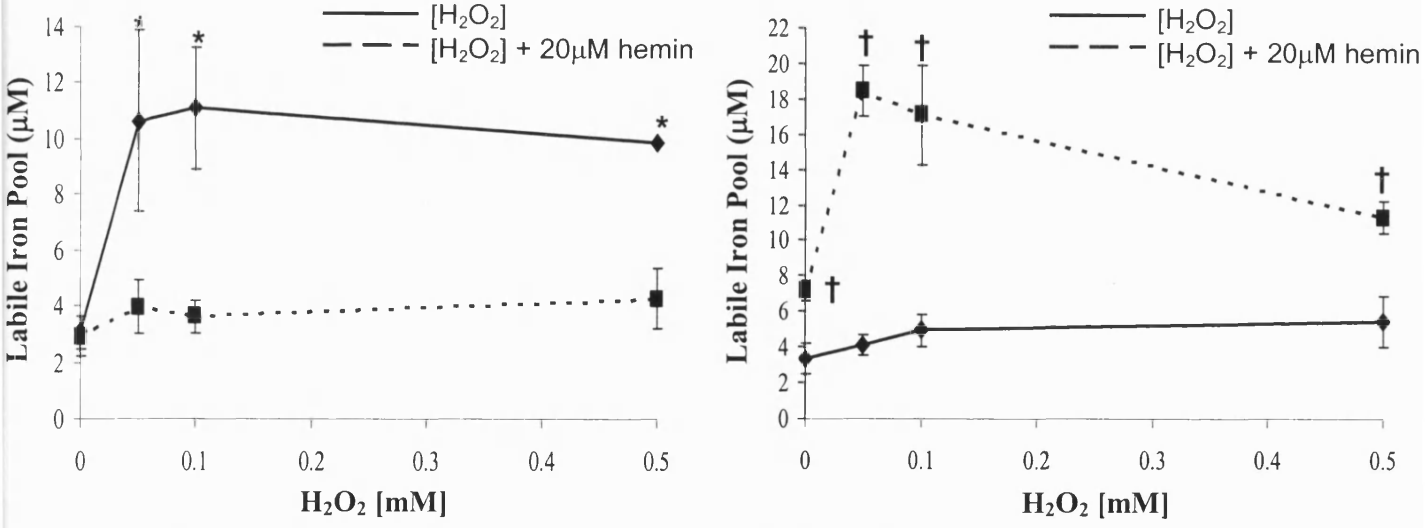



***Figure 3.16*:** Effect of $H_2O_2$ on the concentration of labile iron pool in both parental (A) and $H_2O_2$ resistant (B) Jurkat cells. Both cell lines were treated with 20 μM hemin for 18 h prior to $H_2O_2$ treatment. The Calcein assay was performed immediately after $H_2O_2$ treatment
Mean ± SD (n =3-6)
* : $p<0.05$, significant difference between $H_2O_2$ alone and $H_2O_2$ + hemin, in parental cells
† : $p<0.05$, significant difference between $H_2O_2$ alone and $H_2O_2$ + hemin, in $H_2O_2$ resistant cells

### 3.6 The role of Ferritin in response to oxidative stress

Several studies have linked the higher intracellular level of Ft to higher resistance of cells to oxidative damage (Lin and Girrotti, 1997; Applegate *et* al, 1995). In addition, studies by Epsztejn *et al* (1999) demonstrated that overexpression of the H-Ft in murine erythroleukemia cell lines reduced the formation of ROS and protected cells against cellular damage by exposure to $H_2O_2$.

However, recently it has been shown that Ft might act as a pro-oxidant since both UVA (Pourzand *et* al, 1999) and $H_2O_2$ (unpublished data, this laboratory) could trigger the immediate and dose-dependent proteolytic degradation of Ft molecules and release the iron. Furthermore, it appears that modulation of the basal level of Ft in cells could directly influence both the level of oxidant-induced LIP release and increased susceptibility of cells to necrotic cell death (unpublished data, this laboratory).

We therefore measured the basal Ft levels in both parental and $H_2O_2$ resistant cells, using both ELISA (see section 2.8) and immunoprecipitation methods (see section 2.9).

The results of Ft ELISA (**Table 3.6**) showed no significant difference in Ft levels between parental (77.16 ± 10.75) and $H_2O_2$ resistant cell lines (82.32 ± 16.39). However since the ELISA kit used in this study is known to reflect mostly the level of human L-Ft, we decided to monitor the level of Ft with immunoprecipitation using a human polyclonal Ft antibody following metabolic labelling of both cell lines with 35S-methionine (see section 2.9), as this method is extremely sensitive for evaluation of this large cytosolic protein (see Pourzand *et al*, 1999). For this purpose both parental and $H_2O_2$ resistant cells were first metabolically labelled with $^{35}$S-methionine and then treated with a dose of either 250 kJ/m$^2$ UVA or 500 μM $H_2O_2$ followed by

immunoprecipitation with a human polyclonal Ft antibody. From **Figure 3.17** that shows two representative gels from three independent experiments, it can be observed that Ft basal level is higher in $H_2O_2$ resistant cells as compared to the parental cells. Furthermore in both cell lines immediately after UVA or $H_2O_2$ treatment, no change in the Ft levels is apparent.

*3.6.1* The level of Ft after $H_2O_2$ ± Hemin ± DFO treatments

Several studies have shown that loading of cells with iron sources (e.g. hemin) can cause an increase in intracellular level of Ft. It is also known that iron deprivation in the cells following treatment of cells with an iron chelator (e.g. DFO) could significantly decrease the level of Ft.

In the present study, we assessed the levels of Ft in both parental and $H_2O_2$ resistant cells following 2 and 18 h treatment with either hemin or DFO. The Ft levels were measured with both ELISA (see section 2.8) and immnuoprecipitation methods (see section 2.9). The Ferritin ELISA method (**Table 3.7**) demonstrated that in both cell lines, 2 h hemin treatment did not significantly increase the level of Ft, while incubation of cells for 18 h with hemin increased the Ft level up to 3 fold of the control values in both cell lines. For DFO treatment, the 2 h treatment did not affect the level of Ft in both cell lines. However following 18 h DFO treatment, the intracellular level of Ft decreased up to 2.5 fold of the control values in parental cells and up to 4 fold in $H_2O_2$ resistant cells. These results were also in agreement with the observations obtained from the immunopricipitation method (**Figure 3.18**). First, we confirmed that Ft basal levels are up to 2 fold higher in $H_2O_2$ resistant cells when compared to the parental cells. When both cell lines were treated with hemin for 18h, a 2 fold increase in Ft levels could be observed in both parental and $H_2O_2$ resistant

cells. However, when both cells were treated with DFO Ft synthesis was inhibited in both cell lines.

*Table 3.6:* Assessment of Ft concentration (ng ferritin/mg protein) in both parental and $H_2O_2$ resistant Jurkat cells using an ELISA kit.

| | **[Ferritin] ng ferritin per mg protein** | ***Fold difference*** |
|---|---|---|
| ***Parental cells*** | 77.16 ± 10.75 **NS** | 1 |
| ***$H_2O_2$ resistant cells*** | 82.32 ± 16.39 | 1.1 |

Note: Mean ± SD (n = 3)

NS : $p>0.05$, no significant difference between parental and $H_2O_2$ resistant cells

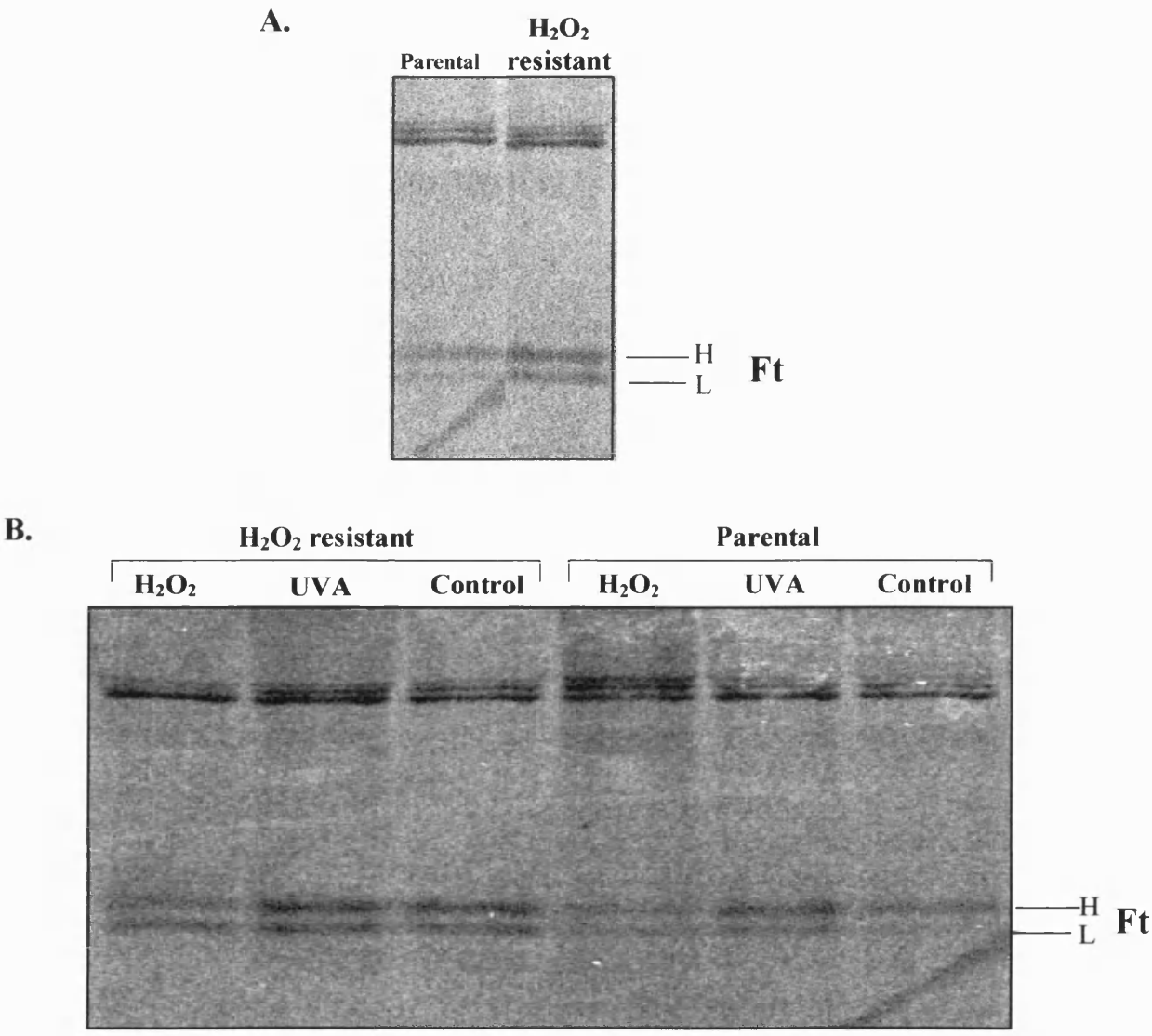



***Figure 3.17***: Effect of UVA or $H_2O_2$ on intracellular level of Ft. $^{35}S$-labeled parental and $H_2O_2$ resistant cells were exposed or not (i.e. in ***A*** and 'Control' in ***B***) to either UVA (250 $kJ/m^2$) or $H_2O_2$ (500 μM) and then immediately lysed and immunoprecipitated with human polyclonal Ft antibody. Both A and B are representative gels chosen from three independent experiments.

*Table 3.7:* The effect of hemin or DFO treatment on Ft concentration in both parental and $H_2O_2$ resistant Jurkat cells as measured by an ELISA kit.

| [Ferritin] ng ferritin per mg protein | | |
|---|---|---|
| | ***Parental cells*** | ***$H_2O_2$ resistant cells*** |
| Control | 77.16 ± 10.75 $^{NS}$ | 82.32 ± 16.39 |
| Hemin $^{2h}$ | 117.003 ± 26.19 | 102.41 ± 33.8 |
| DFO $^{2h}$ | 87.598 ± 15.23 | 77.985 ± 21.12 |
| Hemin $^{18h}$ | 243.59 ± 30.28 * | 248.65 ± 37.79 * |
| DFO $^{18h}$ | 33.01 ± 11.3 * | 18.815 ± 8.78 * |

Note: Both cell lines were treated with 100 μM DFO or 20 μM hemin for 2 and 18 h. the Ft level was assessed in cell lysates using the Ft ELISA kit.
Mean ± SD (n = 3)
NS : $p>0.05$, no significant difference between parental and $H_2O_2$ resistant cells
* : $p< 0.05$, significant difference compared to untreated control

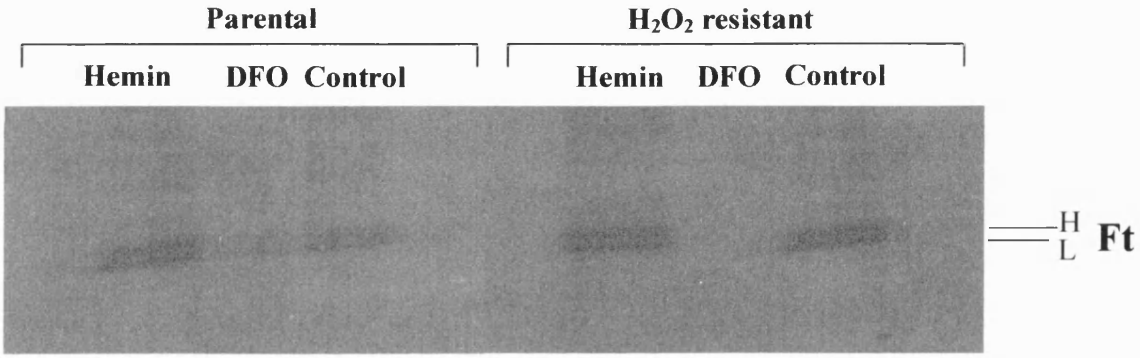



***Figure 3.18*:** Effect of DFO or Hemin on intracellular level of ferritin. $^{35}$S-labeled parental and $H_2O_2$ resistant cells were exposed or not to either DFO (100 μM) or Hemin (20 μM) and then immediately lysed and immunoprecipitated with human polyclonal Ft antibody. This is a representative gel chosen from three independent experiments.

## 3.7 The role of HO-1 in resistance of cells to $H_2O_2$

Much evidence was accumulated, both *in vitro* and *in vivo,* that HO-1 participates in cellular defence mechanisms against oxidative stress. Keyse and Tyrrell (1989) were the first to identify HO-1 as the 32kDa stress protein induced in human cells by a variety of oxidising agents including UVA and $H_2O_2$. Further studies reported that UVA radiation and $H_2O_2$ induce strong transcriptional activation of the HO-1 gene (Vile and Tyrrell, 1993) which eventually leads to a HO-1 dependent increase in Ft and a consequent decrease of the pro-oxidant state of the cells (Vile *et al*, 1994).

The differential response of Jurkat cell lines to $H_2O_2$ cytotoxicity and labile iron release following hemin treatment, prompted us to investigate whether differences in expression/activity of HO-1 could play a role in this phenomenon, since hemin is a known substrate of the HO-1 enzyme.

### *3.7.1* HO-1 expression at the protein level

The basal and hemin stimulated levels of HO-1 expression was first assessed by western blotting (see section 2.10). For this purpose, parental and $H_2O_2$ resistant cells were incubated with 20 μM hemin for 2, 4, 6, 8, 12 and 18 h prior to analysis with western blotting using a polyclonal HO-1 antibody (see Chapter 2.10). The western blot shown in **Figure 3.19** is a representative blot from four independent experiments. Although because of cross hybridisation, these blots were not quite clean, we could still consistently observe that firstly the basal level of HO-1 in $H_2O_2$ resistant cells was higher than in parental cells and that secondly following hemin treatment, a significant increase in HO-1 expression could only be seen in the parental cells (**Figure 3.19**). Furthermore in both cell lines, 18 h following hemin treatment, HO-1 expression had returned to around control level.

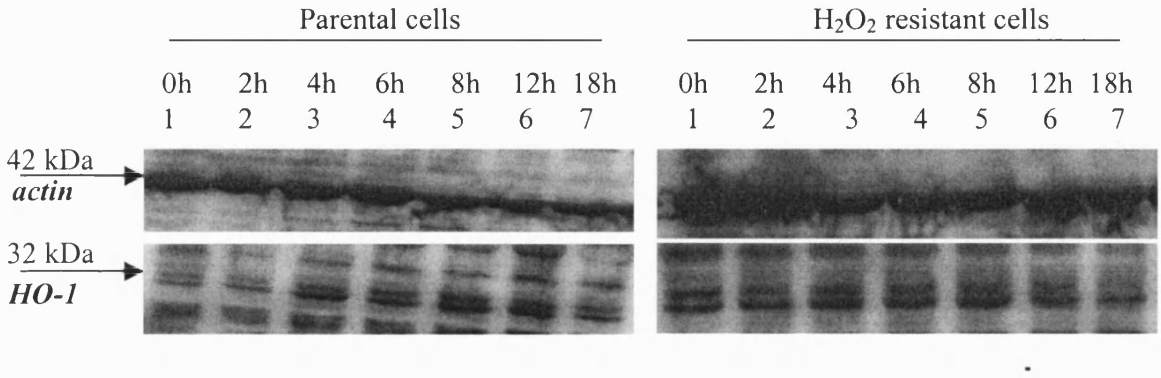



***Figure 3.19*:** Time course of HO-1 expression following treatment of both parental and $H_2O_2$ resistant cell lines with 20 μM hemin. Cells were treated with hemin for various times (lane 1-7) and then analysed by Western blotting using a polyclonal HO-1 antibody. Actin antibody was used as a marker of even loading. This is a representative blot chosen from four independent experiments.

To confirm the results of the western blotting, the expression of HO-1 in hemin treated cells was also followed by flow cytometry using a monoclonal HO-1 antibody. The results (**Figure 3.20**) revealed that the basal level of HO-1 protein in $H_2O_2$ resistant cells was up to 2 fold higher than in parental cells. The latter result was in agreement with the observation made in western blot analysis. Furthermore when both cell lines were treated with 20 μM hemin for various time points, a significant increase in HO-1 expression could only be observed in the parental cells around 2 and 4 h following the treatment. Moreover, although the basal level of HO-1 expression was higher in $H_2O_2$ resistant cells than in parental cells, following hemin treatment no significant increase was observed between the basal and hemin treated $H_2O_2$ resistant cells. These results are consistent with the observations made by the western blot analysis and clearly demonstrate the refractory response of HO-1 expression to hemin treatment in $H_2O_2$ resistant cells.

In parallel to western blot/flow cytometric analyses, the kinetics of labile iron release was also monitored in both cell lines following hemin treatment. The results (**Figure 3.21**) showed that in $H_2O_2$ resistant cells after hemin treatment, the extent of labile iron release was not only higher (up to 4 fold) but also sustained for a longer period than in parental cells. The higher basal level of HO-1 in $H_2O_2$ resistant cells is likely to play a role in higher level of labile iron release following hemin treatment.

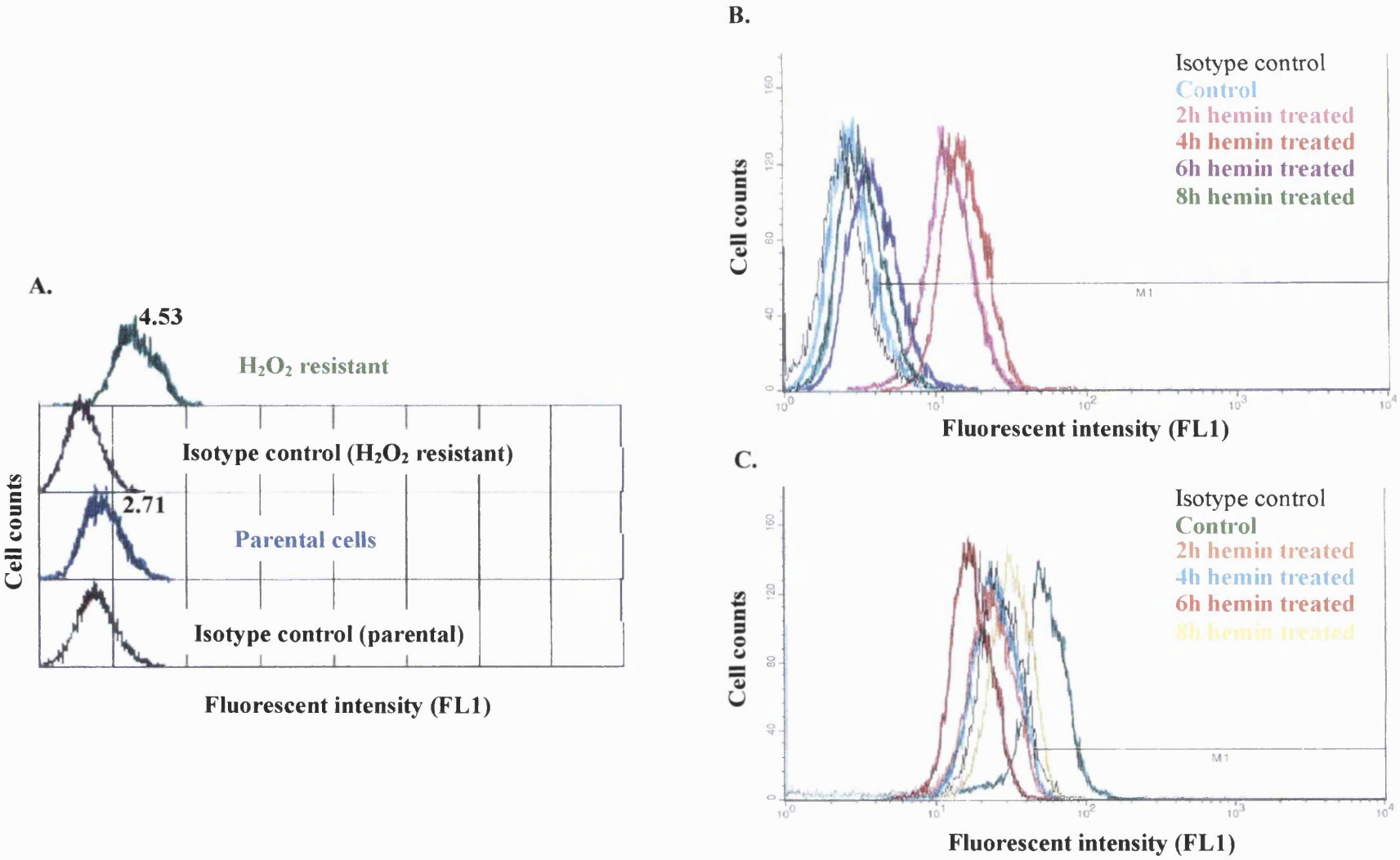



***Figure 3.20***: Flow cytometric analysis of relative increases in HO-1 expression in both parental and $H_2O_2$ resistant cells. Both cell lines were incubated with 20 μM hemin for different time points (2, 4, 6 and 8 h) and then incubated with HO-1 monoclonal antibody as described in section 2.11. A, shows the control levels of both cell lines based on the isotype controls. The median values are provided in bold on the peak of the fluorogram. B and C show the shifts in fluorescence obtained at different time points of hemin treatment in the parental (B) and in $H_2O_2$ resistant (C) cells.

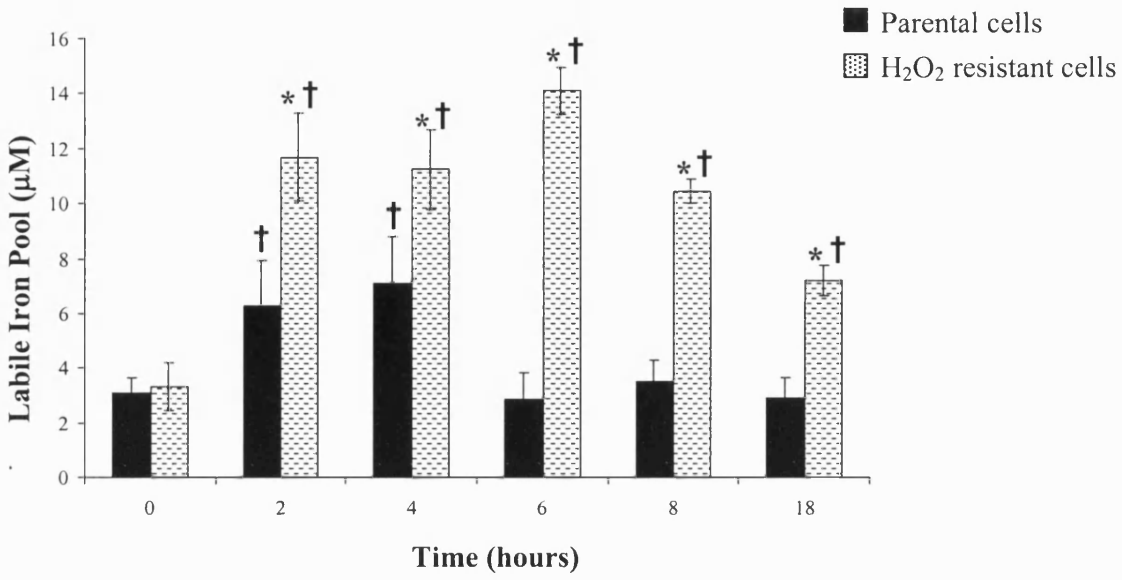



***Figure 3.21*:** Time course of change in LIP levels following teatment of both parental and $H_2O_2$ resistant cells with 20 μM hemin. Cells were treated with hemin for various times (i.e. 0 to 18 h) and then the change in LIP was monitored by Calcein assay. Mean ± SD (n = 6 - 8)
* : *$p<0.05$*, significant difference between normal and $H_2O_2$ resistant cells, at corresponding time points
† : *$p<0.05$*, significantly different from untreated control

*3.7.2* HO-1 expression at the transcriptional level

Since the transcriptional activation of the ho-1 gene is recognised to be a marker of oxidative stress in mammalian cells, we examined the levels of accumulation of ho-1 mRNAs in both parental and $H_2O_2$ resistant cells using both RT-PCR and the newly developed method of real time PCR (Light Cycler).

Despite numerous attempts to optimise the RT-PCR conditions, we failed to obtain a visible band for ho-1 in the agarose gel for either parental or $H_2O_2$ resistant cells at both basal and treated levels. A representative gel is shown in **Figure 3.22**, in which RT-PCR was performed in both parental and $H_2O_2$ resistant cells 4 h following $H_2O_2$ treatment. To show that the PCR conditions and the amplification steps were optimum, the parallel amplification of the ho-1 cDNA was used as a positive control (**Figure 3.22**, lane 6). Furthermore to demonstrate that the lack of amplification bands in the agarose gel was not due to RNA degradation that may affect the performance of RT-PCR, the cDNAs were also amplified with βactin primers. As shown in **Figure 3.22,** βactin amplification was consistent and successful for all the samples.

To analyse the level of ho-1 mRNA accumulation with a more sensitive method, an attempt was made to use the real time PCR technique using the DNA binding dye SYBR Green I (section 2.15) in the Roche Ltd developed Light Cycler amplifier. For this purpose, both parental and $H_2O_2$ resistant cells were treated with 500 μM $H_2O_2$ and then analysed by real time PCR 2, 4, 6, 8 and 18 h following the treatment. The results (**Figure 3.23)** showed that firstly the basal level of ho-1 mRNA is 2 fold higher in $H_2O_2$ resistant cells than in parental cells. Furthermore, 2 h following $H_2O_2$ treatment, the ho-1 cDNA levels increase up to 1.6 fold and then quickly returns to around control values from 4 h to 18 h post treatment time points. In the case of

parental cells, there was no significant increase in ho-1 cDNA levels when monitored up to 18 h post treatment time.

To compare the response of both cell lines to $H_2O_2$ following hemin treatment, the cells were also treated with 20 μM hemin prior to $H_2O_2$ treatment with a dose of 500 μM. The results revealed no difference on the ho-1 expression in either parental or $H_2O_2$ resistant cells.

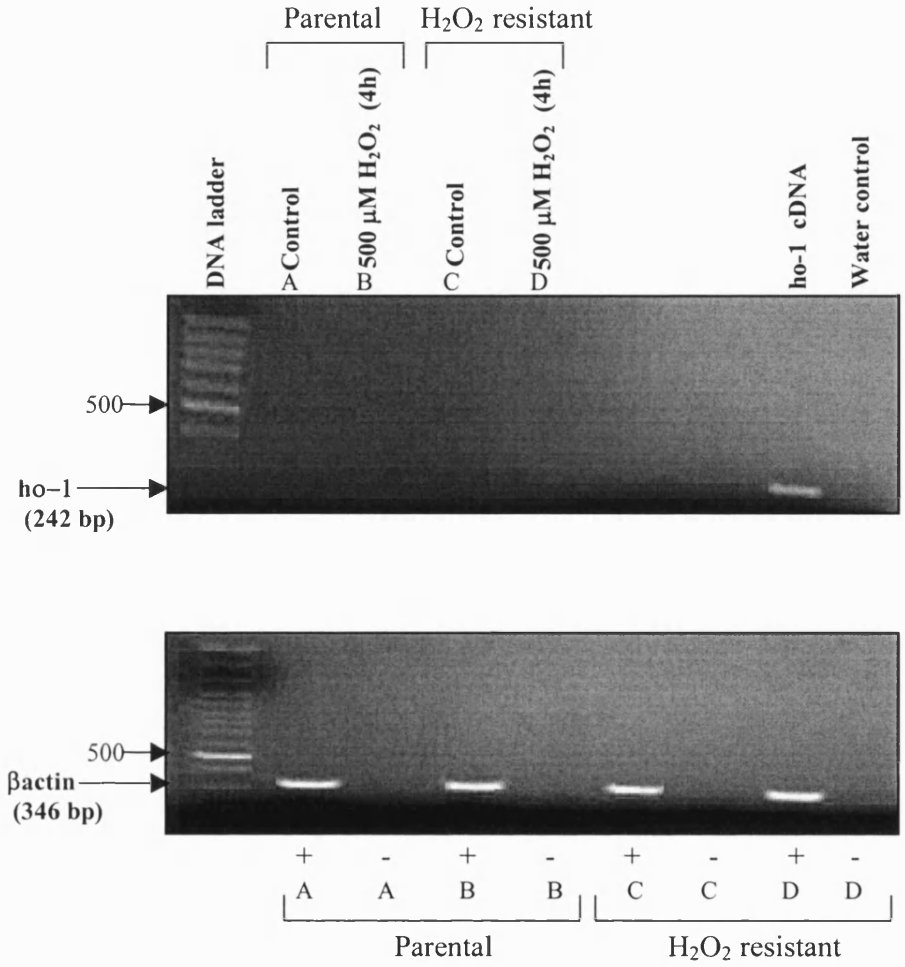



***Figure 3.22*:** Analysis of the amplified products of RT-PCR in untreated control (Control) and 4 h following $H_2O_2$ treatment in both parental and $H_2O_2$ resistant cells. The PCR products were run on a 2% agarose gel stained with ethidium bromide. Samples were either amplified with ho-1 or βactin primers giving products of at 242 bp and 346 bp, respectively. For βactin analysis negative controls (-) included no RT step. The ho-1 cDNA was used as a positive control.

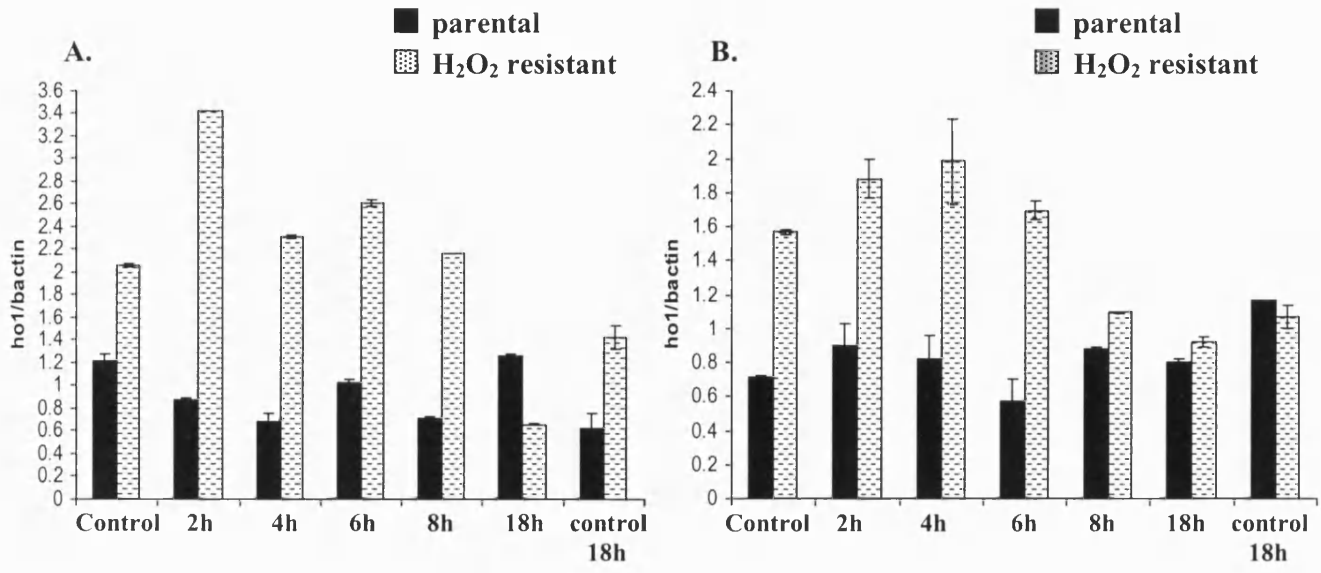



***Figure 3.23***: Analysis of the ho-1 and βactin expression, using the Lilght Cycler. In A, both cell lines were treated with 500 μM $H_2O_2$ and incubated for 2, 4, 6, 8 and 18 h prior to the analysis. In B, both cell lines were treated overnight with 20 μM hemin and then exposed to 500 μM $H_2O_2$ and analysed 2, 4, 6, 8 and 18 h following $H_2O_2$ treatment. The figures A and B represent the ratio of ho-1/βactin expression as calculated using the standard curve analysis (section 2.15).
Mean ± SD (n = 4)
No significant difference was observed between parental and $H_2O_2$ resistant cells in both treatments.

## 3.8 The correlation between oxidative stress and MDR

*3.8.1* Characterisation of the expression of mdr genes

Expression of the mdr-1 gene has been shown to be associated with drug resistance. Studies on expression of mdr-1 mRNA have indicated that the mdr-1 gene encodes for the multidrug resistant transporter, PgP. An important study by Epsztejn *et al* (1999) indicated that overexpression of the H-Ft subunit in a murine erythroid leukaemia cell line can reduce the response of cells to oxidative stress by downregulating the level of the labile iron pool in the cells and inducing the MDR phenotype which involves increased levels of mdr1 mRNA and protein (PgP) as well as drug transport and drug toxicity.

To investigate whether there is a correlation between the increased resistance of cells to $H_2O_2$ and the induction of the MDR phenotype, we performed a series of RT-PCR reactions using specific primers designed to amplify the MDR-related specific genes mdr-1, mxr and mrp-1.

The study of the expression of mdr-1 gene involved treatment of both parental and $H_2O_2$ resistant cells with non-toxic doses of vinblastin (50 nM) for various time points (6, 18 and 24 h), since vinblastin has been shown to significantly induce the increase in the mdr-1 levels in various tumour cell lines. To ensure that both PCR conditions and the quality of prepared RNA were optimum as positive controls, two different concentrations of mdr-1 cDNA (2 and 5 μM) were used. Furthermore the amplification of samples with β-actin primers was used to demonstrate the quality of the prepared RNA and the lack of RNA degradation in the samples. Also, the RT negative samples were amplified in parallel to check the presence of any genomic contamination in the samples. The results (**Figure 3.24)** showed no detection of the mdr-1 gene either for the control or treated samples indicating that mdr-1 gene

expression is quite low in these cell lines and is unlikely to play a role in the increased resistance of cells to $H_2O_2$.

In addition to mdr-1, the level of expression of mrp-1 and mxr genes was also investigated by RT-PCR using cDNA samples obtained after treatment of both cell lines with the non-toxic dose of 20 μM doxorubicin. The agarose gels (**Figure 3.25** and **Figure 3.26**) showed no detectable band for either the mrp (**Figure 3.25**) or mxr (**Figure3.26**) gene in either the control or the treated samples analysed. These results were similar to the data obtained with the mdr-1 gene. Taken together these data strongly indicated that expression of these MDR-related genes are quite low in these cell lines and therefore their involvement in the higher resistance of cells to $H_2O_2$ is unlikely.

However because the MDR-related genes are a growing family, it could be postulated that the above RT-PCR analyses is not broad enough to represent the entire MDR genes involved, as several new subfamily members have been recently identified for both mdr and mrp genes (Litman *et al*, 2000). Furthermore since the above analyses were performed at the transcriptional level, it was relevant to evaluate the expression of these genes at the protein level. However instead of analysing all candidate proteins with specific antibodies, we focussed on achieving general information about the involvement of any possible MDR-related protein with efflux activity in increased resistant of cells to $H_2O_2$. To this regard, we performed a series of drug efflux assays in both parental and $H_2O_2$ resistant Jurkat T cell lines by flow cytometry using the fluorescent dye $DiOC_2(3)$ that has been shown to be a common substrate for the mammalian ABC transporter proteins involved in MDR phenomenon such as PgP, MRP and MXR.

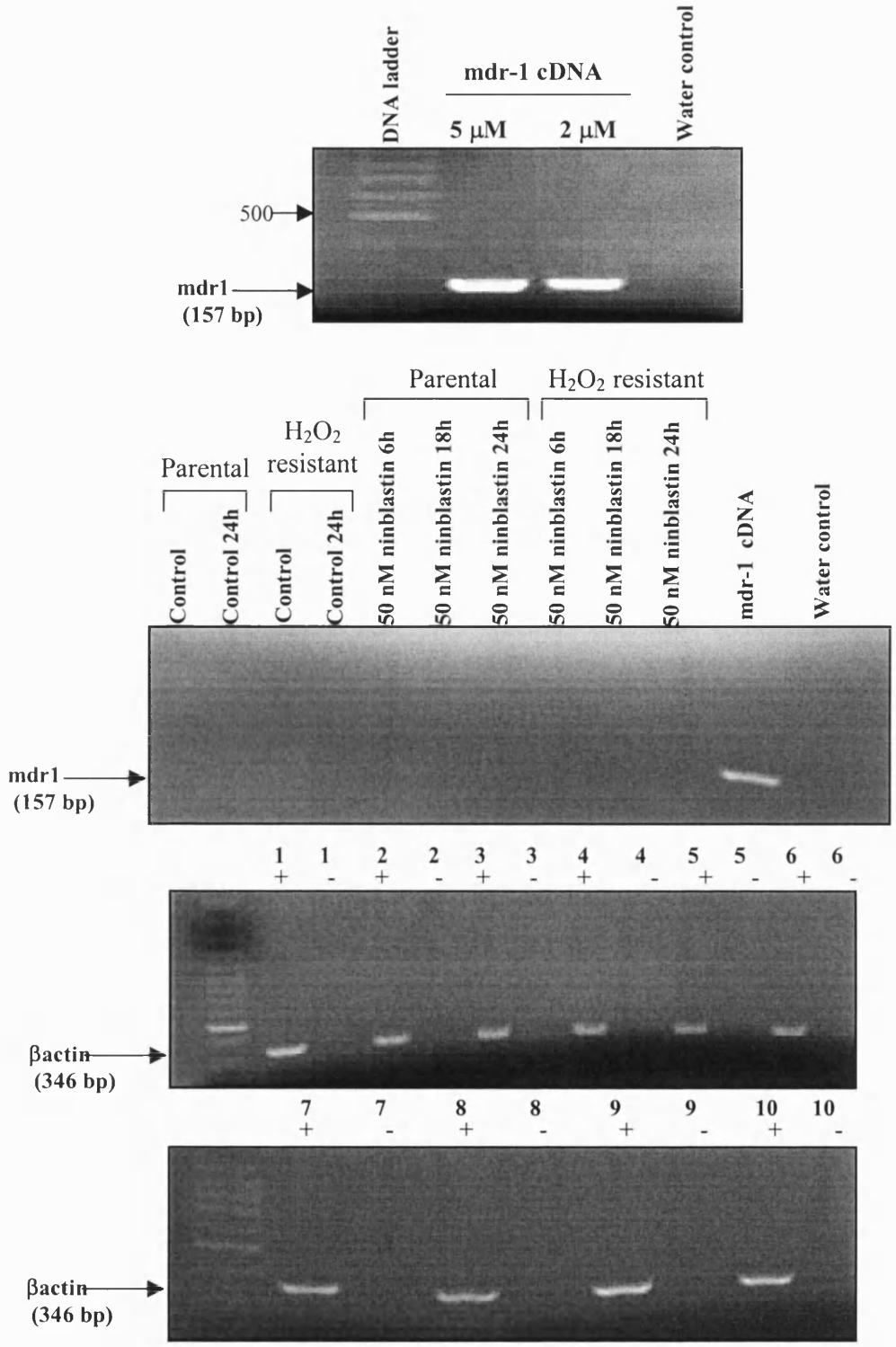



***Figure 3.24*:** Analysis of the amplified products of RT-PCR in parental and $H_2O_2$ resistant cells 6, 18 and 24 h following vinblastin treatment. The amplified samples were loaded on a 2% agarose gel stained with ethidium bromide. Samples were either amplified with either mdr-1 or βactin primers giving products of at 157 bp and 346 bp, respectively. For βactin analysis negative controls (-) included no RT step and numbers 1 to 10 represent the samples as shown on the gel for mdr-1 detection. The mdr-1 cDNA was used as a positive control and as shown on the first-top gel 2 and 5 μM were initially tested before the experiment.

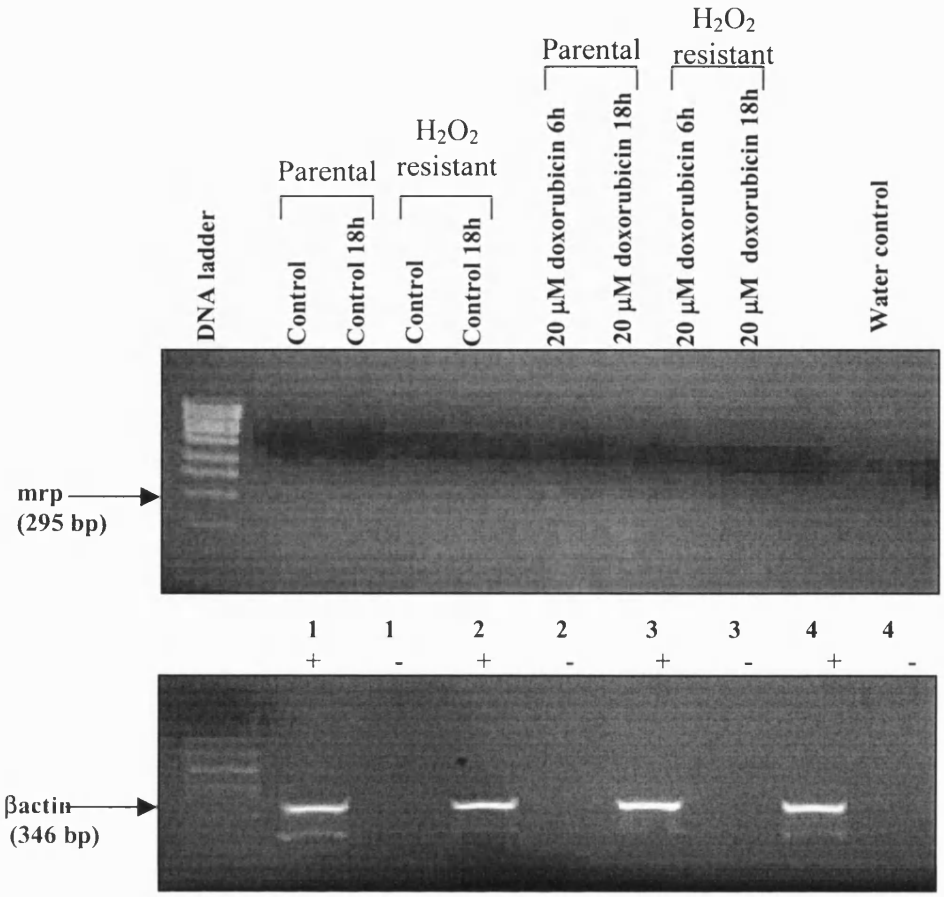



***Figure 3.25*:** Analysis of the amplified products of RT-PCR in parental and $H_2O_2$ resistant cells 6, and 18 h following treatment with doxorubicin. The amplified samples were loaded on a 2% agarose gel stained with ethidium bromide. Samples were amplified with either mrp or βactin primers giving products of at 295 bp and 346 bp, respectively. For βactin analysis negative controls (-) included no RT step and numbers 1 to 5 represent the samples as shown on the gel for mrp detection of the doxorubicin treated samples in the order shown on the top gel.

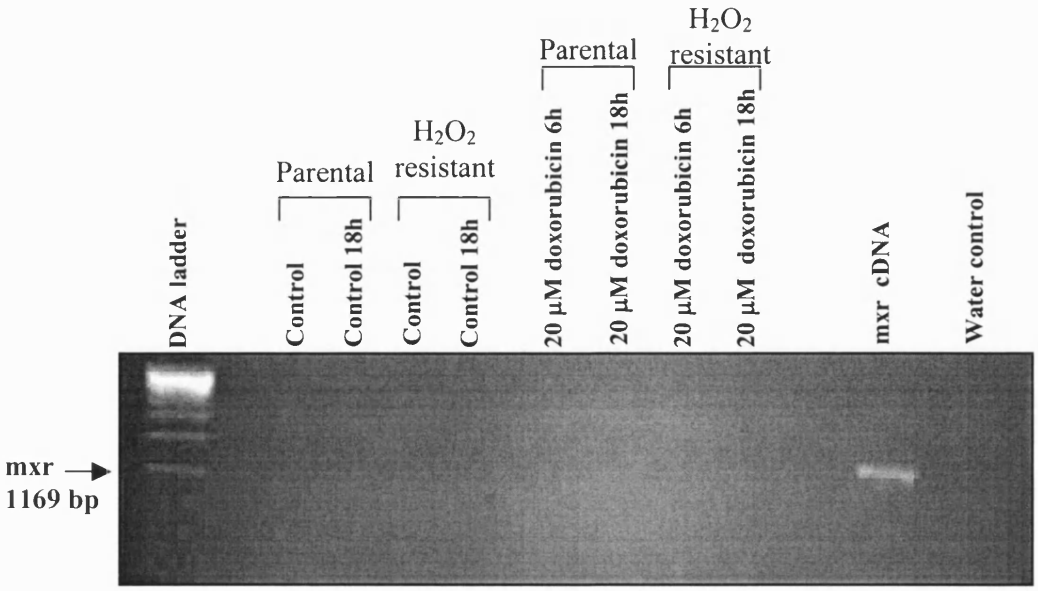



***Figure 3.26*:** Analysis of the amplified products of RT-PCR in parental and $H_2O_2$ resistant cells 6 and 18 h following treatment with doxorubicin. The amplified samples were loaded on a 2% agarose gel stained with ethidium bromide. Samples were either amplified with mxr primers giving product of 1169 bp. The mxr cDNA was used as a positive control.

*3.8.2* Efflux assay: Example of PgP

The PgP has been widely described as a cationic pump that when overexpressed induces MDR phenotype in various cancer cell lines. In the present work, we analysed the possible mechanism underlying the role of PgP function in the increased susceptibility of both parental and $H_2O_2$ resistant cells to oxidative damage. In particular, we compared the results obtained between Jurkat T cell lines used in this thesis and a well-established cell model for MDR phenotype i.e. the parental CEM lymphoid cell line (CEM) and its MDR variant ($CEM/VLB_{100}$). For this purpose the baseline fluorescence signal of $DiOC_2(3)$ efflux and inhibition of efflux by verapamil was compared to determine the level of PgP efflux activity. **Figure 3.27** represents the flow cytometric analysis of the PgP function in the CEM cell lines. These data clearly demonstrate an active efflux of the PgP pump in the $CEM/VLB_{100}$ cells at both 30 and 90 min of incubation time points, while in the CEM cells, no increase is observed in the fluorescence signal after the addition of the PgP inhibitor, verapamil, in agreement with observations made by other studies (Feller *et al*, 1996; Ganeshaguru *et al*, 2002). Interestingly in both Jurkat T cell lines (**Figure 3.28**) there was no increase in the fluorescence after the addition of verapamil indicating that there is no PgP surface expression either at 30 or 90 min of incubation.

Finally, the fact that the Jurkat T cell lines retain the fluorescence dye $DiOC_2(3)$ as efficiently as parental CEM cell line, strongly suggests that none of the suspected MDR proteins are overexpressed in these cells. In other words, it appears that the higher resistance of $H_2O_2$ resistant Jurkat T cell line to H2O2 is not linked to the development of MDR phenomenon in these cells.

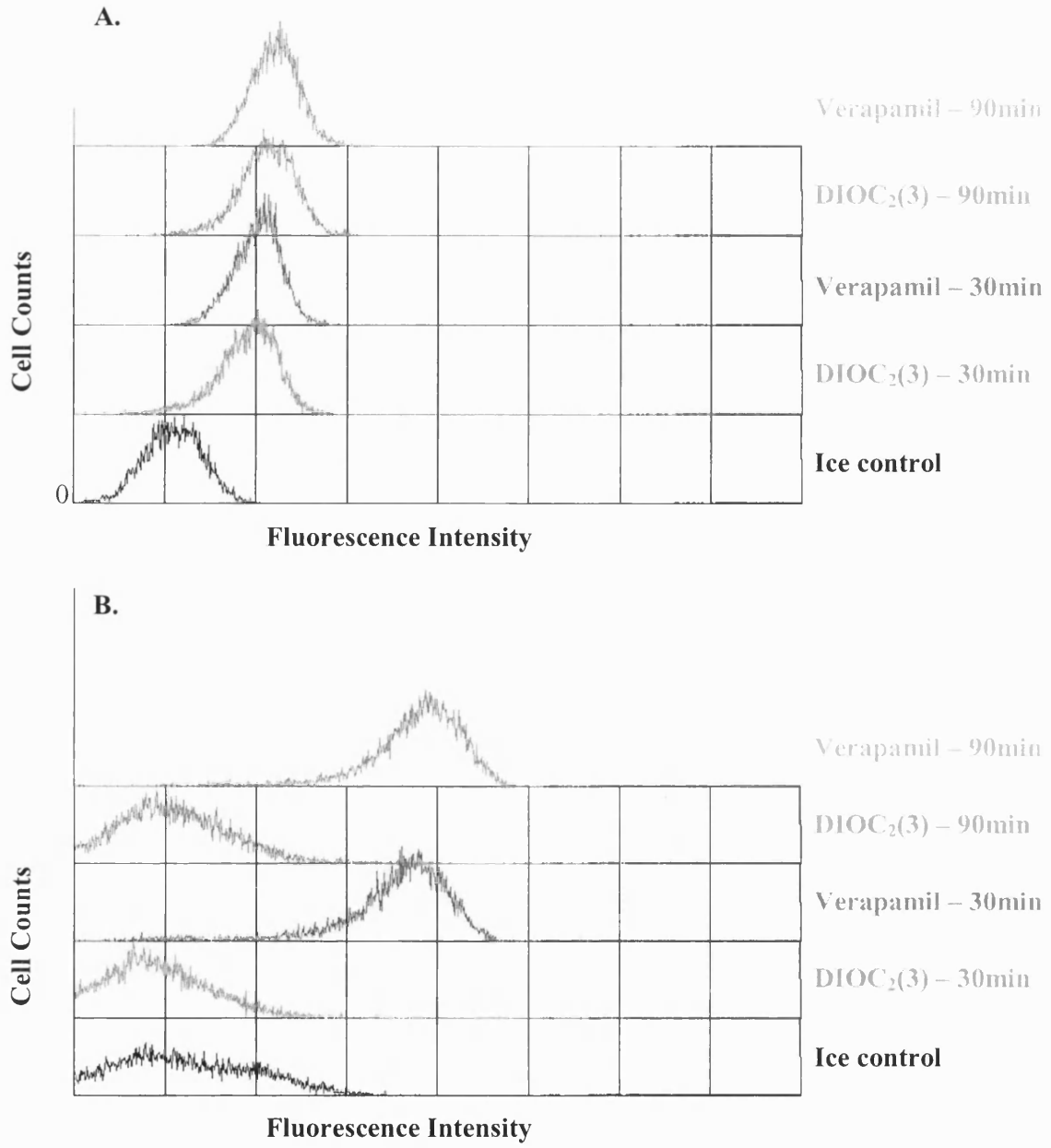



***Figure 3.27***: Modulation of efflux of the dye $DiOC_2(3)$ by verapamil in both CEM (A) and CEM/$VLB_{100}$ (B) cell lines. Analysis was performed after 30 and 90 min of $DiOC_2(3)$ dye loading in the presence or absence of the PgP inhibitor, verapamil, using FACScanflow cytometer.

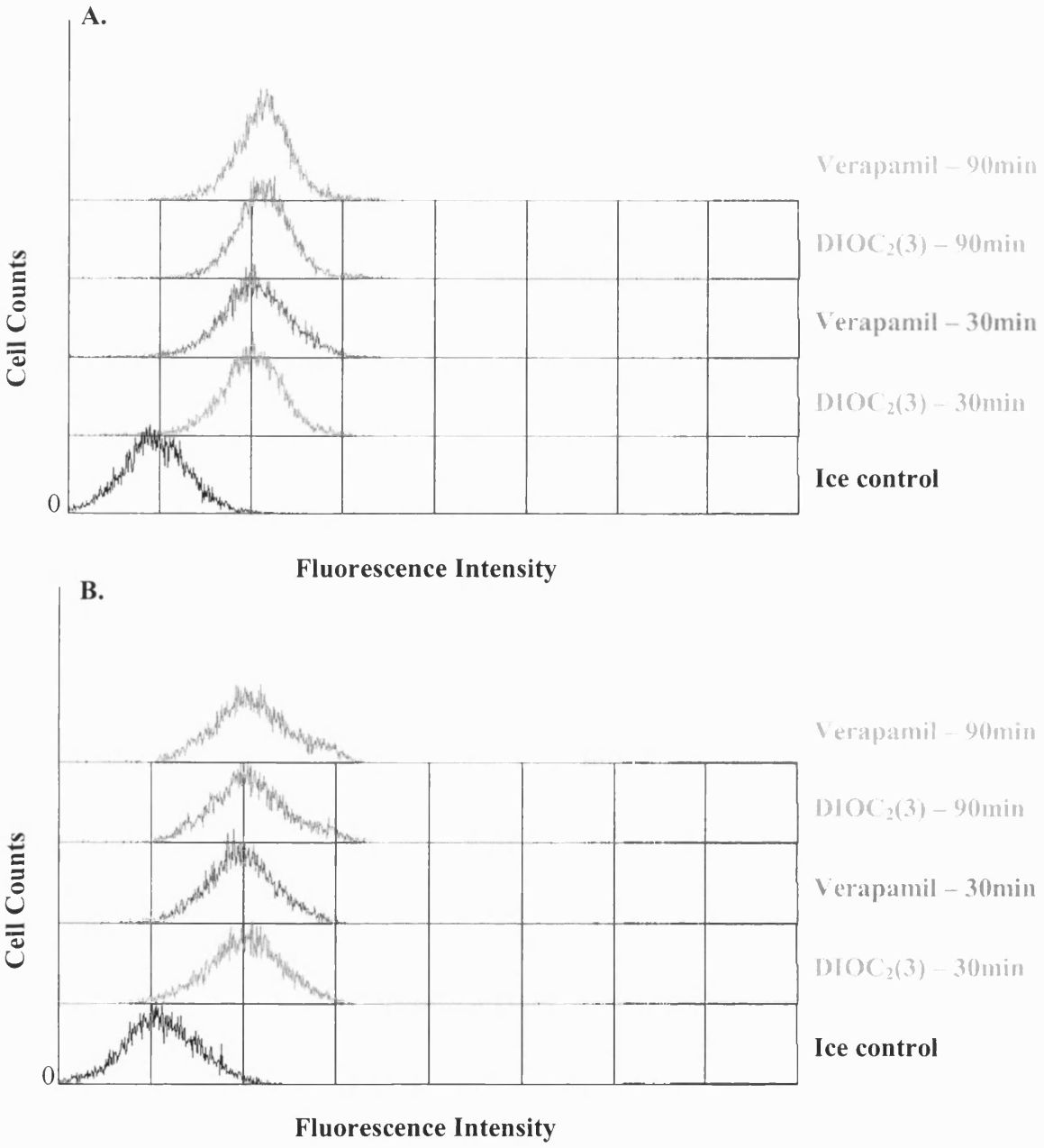



***Figure 3.28*:** Modulation of efflux of the dye $DiOC_2(3)$ by verapamil in both parental (A) and $H_2O_2$ resistant (B) cells lines. Analysis was performed after 30 and 90 min of $DiOC_2(3)$ dye loading in the presence or absence of the PgP inhibitor, verapamil, using FACScanflow cytometer.

# CHAPTER 4

# DISCUSSION

Exposure to ROS is a common threat in mammalian cells. Oxidative stress occurs under circumstances where the amount of oxidants exceed a certain threshold level leading to the induction of oxidative damage. Recent studies in this field clearly demonstrate that the response of cells to either an acute (single high dose) or chronic (repeated low doses) exposures to oxidizing agents is quite different. Indeed it has been shown that chronic exposure of cells to low doses of oxidants provokes the development of an adaptive response in cells as a result of induction of a series of intracellular antioxidant pathways that differs quite sharply from that of acute exposure. The adaptive response triggered by chronic exposures allows the cells to withstand high toxic doses of the oxidizing agent that would otherwise be lethal to the cells.

Recently a number of studies have provided evidence for a possible link between the resistance of cells to high toxic concentrations of oxidising agents (such as $H_2O_2$) and the development of MDR phenotype (see Introduction, section 1.10). Most of these studies demonstrate that tumor cells that exhibit the MDR phenotype are also resistant to oxidizing agents such as $H_2O_2$ or UVA. However, to date no study has investigated whether adaptation of cells to high doses of oxidising agent would induce the MDR

phenotype. Moreover there are studies that link the overexpression of H- or L-Ft in cells both to the development of MDR phenotype and to the higher resistance of cells to oxidising agent. However, to date no study has verified whether cells exhibiting resistance to oxidative stress would necessarily possess higher levels of expression of Ft and MDR-related proteins.

To answer these questions, in the present study, we used a cell model of parental and $H_2O_2$ resistant Jurkat T cell lines. Our investigation involved the characterisation of these cell lines in terms of intracellular anti-oxidant defence mechanism by comparing the level of expression of catalase, GSH-Px, GSH, Ft and HO-1 between parental and $H_2O_2$ resistant cells. Furthermore, the role of labile iron and heme in modulating the resistance of cells to $H_2O_2$ was investigated. To investigate the possible link between the development of MDR phenotype and resistance to oxidative stress, the expression of key MDR-related genes were also monitored and compared between the two cell lines.

The role of antioxidant molecules/enzymes:

It is assumed that high antioxidant status of the cell prevents $H_2O_2$ from travelling far through the cell. Under most circumstances, cellular antioxidant systems remove $H_2O_2$ efficiently. Since catalase, GPx and GSH could detoxify $H_2O_2$ within the cells, we hypothesised that these enzymes/molecules could also contribute to the protective responses of $H_2O_2$ resistant cells against oxidative stress. Our results clearly demonstrated that GPx activity is significantly higher in $H_2O_2$ resistant cells than in parental cells. Furthermore, it was found that the basal level concentration of GSH is also considerably higher in $H_2O_2$ resistant cells compared to parental cell line. These data

strongly suggest that both higher GPx activity and GSH levels could be part of the adaptive response of cells that contribute to higher resistance of cells to $H_2O_2$. However catalase appears to play a minor role in this response. One explanation for the relative importance of GPx and GSH and not of catalase in the resistance of cells to $H_2O_2$ may be due to their intracellular location. Catalase enzyme is located in peroxisomes and its access to cytosolic $H_2O_2$ is limited. However GPx, unlike catalase, is mainly present in the cytosol and requires GSH to complete the catalytic cycle so it is likely that in $H_2O_2$ resistant cells, $H_2O_2$ is reduced at higher levels by GPx than catalase.

The role of labile iron in increased susceptibility of cells to necrotic cell death:

Although several studies have shown that iron loading could sensitise cells to iron-mediated oxidative damage, little is known about how 'basal' intracellular LIP levels could influence the natural sensitivity of cells to oxidants. Recent unpublished data from this laboratory provide a strong link between the basal intracellular level of LIP in skin cells and the susceptibility of cells to UVA radiation. Accordingly, modulation of the basal or 'UVA-induced' levels of LIP appears to directly influence the extent of necrotic cell death in skin cells (unpublished data, this laboratory). In the present study it was found that necrosis is the predominant mode of cell death induced by $H_2O_2$ and UVA in both cell lines, as monitored by both Annexin V/PI and Apoglow assays. However, the $H_2O_2$ resistant cell line was considerably more resistant to $H_2O_2$-mediated necrotic cell death than the parental cell line.

Since $H_2O_2$ is also known to trigger the immediate release of labile iron, it was relevant in this study to investigate whether adaptation of $H_2O_2$ resistant cells to a high

concentration of $H_2O_2$ would influence this phenomenon. The results demonstrated that although the basal level of LIP in both parental and $H_2O_2$ resistant cell lines is similar, the $H_2O_2$-mediated release of iron in $H_2O_2$ resistant cells occurs to a lesser extent than in parental cell line. Furthermore, the lower level of labile iron release following $H_2O_2$ treatment of $H_2O_2$ resistant cell line was coinciding with the lower level of necrotic cell death in this cell line. Taken together these findings strongly suggest that cellular resistance to $H_2O_2$ is tightly associated to intracellular level of LIP and that the "induced" rather than the "basal" level might be responsible for the increased susceptibility of cells to oxidative stress.

Interestingly, the exposure of both parental and $H_2O_2$ resistant cells to UVA promoted: a) similar levels of labile iron release and b) percentage survival, consistent with the notion that $H_2O_2$ and UVA may exert their effect on cells via different oxidative mechanisms.

To study to what extent the intracellular LIP level plays a role in the resistance of cells to $H_2O_2$, both cell lines were incubated with DFO (iron chelation) for 18 h prior to $H_2O_2$ treatment. DFO as an iron chelator is known to deplete intracellular iron levels by either inhibiting Ft synthesis via IRP or by stimulating Ft degradation, consequently preventing oxidative stress (Konijn *et al*, 1999). Previous findings by Lin and Girroti (1993) have identified intracellular level of 'redox active iron' as an endogenous pro-oxidant and demonstrated that chelation of iron with DFO could significantly protect the leukemic cells against Merocyanine 540-mediated oxidative damage. Treatment with DFO has also been shown to completely abolish the UVA-induced LIP release in skin cells, thereby protecting the cells against both mitochondrial membrane damage and

necrotic cell death (unpublished data, this laboratory). In the present study, DFO treatment of normal and $H_2O_2$ resistant cells protected cells against $H_2O_2$- and UVA-mediated damage. These findings are also supported by the assessment of LIP levels in both parental and $H_2O_2$ resistant cells that showed that DFO completely abolishes both the 'basal' and 'induced' level of LIP. Depletion of LIP levels in both cell lines is also consistent with the decrease in Ft levels, indicating that DFO operates by inhibiting Ft synthesis and therefore protects both parental and $H_2O_2$ resistant cells against oxidative damage. Finally our findings are entirely in agreement with the studies reporting that DFO protects cells against the cytotoxic effects of various oxidising agents (see Introduction, section 1.7).

The source of labile iron release in $H_2O_2$-treated Jurkat cell lines

In 1999, Pourzand *et al* clearly demonstrated that the UVA-induced increase in labile iron originates mainly from an "early" degradation of Ft in combination with "intermediate" HO-1-mediated heme breakdown. Accordingly, *in vivo* studies by Cairo and co-workers (Cairo *et al*, 1995; Tacchini *et al*, 1997) have also indicated that oxidative stress down regulates IRP and expands the level of free iron presumably via early proteolysis of Ft and 'intermediate' HO-1 mediated heme breakdown in rat livers. Furthermore, Hentze and Pantopoulos (1995) demonstrated that $H_2O_2$ treatment of cultured murine fibroblasts inhibited Ft synthesis as monitored by immunoprecipitation assay. However, in the present study we failed to demonstrate the early modulation of Ft by $H_2O_2$ in both cell lines. The lack of Ft degradation in these cell lines raises the question as to the source of the labile iron release following $H_2O_2$ treatment of cells.

Much experimental evidence has indicated that there are other potential sources of iron responsible for the increased labile iron release. One such source is the heme that is released from microsomal hemoproteins immediately after UVA or $H_2O_2$ treatment of human skin fibroblasts (Kvam *et al*, 1999). In addressing this issue, Balla *et al* (1992) showed that hemin (1h treatment) made cells more sensitive to lethal effects of $H_2O_2$ and this sensitivity decreased when cells were challenged with hemin for longer time (16-20 h) since cells appeared to develop enhanced resistance to oxidants. Furthermore recent studies from this laboratory using an HO overexpressing cell line have demonstrated that the release of labile iron from heme via HO can cause transient hypersensitivity to oxidative UVA radiation (Kvam *et al*, 2000). Additionally, unpublished data from this laboratory provide strong evidence that hemin treatment of cells prior to UVA radiation could dramatically increase the level of UVA-induced labile iron release and the level of UVA-mediated necrosis (unpublished data). Interestingly, our current findings demonstrated that hemin pre-treatment increases on the one hand the resistance of parental cells to $H_2O_2$-induced necrotic cell death and on the other hand renders the $H_2O_2$ resistant cells more sensitive to cytotoxic effect of $H_2O_2$. To investigate the differential effect of hemin prior to $H_2O_2$ treatment, the LIP levels were assessed. The data showed that hemin induces an increase in basal and $H_2O_2$-induced LIP levels following $H_2O_2$ treatment in $H_2O_2$ resistant cells but not in parental cells. These findings concur with the view that hemin increases the level of potentially harmful labile iron via heme oxygenase which in turn exacerbates the oxidative damage in cells leading to increased level of necrotic cell death in $H_2O_2$ resistant cells. This assumption is further strengthened by the observation that the basal level of HO-1 protein in $H_2O_2$ resistant cell line is two-fold

higher than in parental cells and this should almost certainly contribute to higher rate of hemin breakdown and the consequent increase in both 'basal' and '$H_2O_2$-induced' LIP in $H_2O_2$ resistant cell line.

It is well known that HO-1 is highly induced by oxidative stress in order to remove excess heme from the cell. Vile and Tyrrell (1993) have also reported that in human skin fibroblasts, an increase in Ft coupled with HO synthesis after UVA radiation, is directly linked to ROS formation, secondary to iron release by HO. It has also been shown by Lin and Girotti (1998) that hemin treatment results in elevated H-Ft levels in combination with an early increase in HO-1 levels. They concluded that most of the iron is sequestered in Ft that is being activated after HO-1 induction and thereby intracellular iron levels decrease and cells enhance hyperresistance to oxidising agents. In the present study we found that although HO-1 basal level is higher in $H_2O_2$ resistant cell line, the HO-1 is not induced following $H_2O_2$ or hemin treatment. This phenomenon that is known as refractoriness has also been previously observed in both human skin fibroblasts cells exposed to UVA irradiation 24-48 h following the first insult (Noel and Tyrrell, 1997) or in keratinocytes that possess higher basal HO activity but not inducible HO-1 expression (Applegate *et al*, 1995). Interestingly the refractoriness response of induced HO-1 expression to re-induction has also been shown by hemin or a combination of hemin and $H_2O_2$ treatment (Noel and Tyrrell, 1997). Several studies have also shown that chronic exposure of cells to oxidizing insults could induce the HO-1 refractory response (Reeve and Domanski, 2002; McCourbey and Maines, 1994).

In addition to LIP, we have also investigated whether differences in Ft basal or induced levels might play a role in the differential susceptibility of cells to $H_2O_2$. Ferritin

is an iron storage protein and it has alternatively been seen as a potentially harmful iron donor (Balla *et al*, 1992; Vile and Tyrrell, 1993; Vile *et al*, 1994; Lin and Girotti, 1998). Our results demonstrated that the basal level of Ft in $H_2O_2$ resistant cells is considerably higher than in parental cells and this should almost certainly contribute to the higher resistance of these cells to oxidative stress. The results obtained following hemin treatment further demonstrate that hemin induces Ft synthesis in both cell lines. However it appears that in parental cell line the Ft synthesis induction is mostly related to an HO-1 coupled response whereas in $H_2O_2$ resistant cells, because of lack of induction of HO-1, the Ft induction is likely to occur mainly via IRP-1 regulatory response. Indeed because of higher accumulation of labile iron in $H_2O_2$ resistant cells following hemin treatment it is thought that IRP-1 downregulation would lead to induction of Ft synthesis. Furthermore, since in $H_2O_2$ resistant cells iron loading results in a constant increase in the LIP levels that remains stable even after $H_2O_2$ treatment, it is likely that downregulation of IRP-1 is also sustained for longer period leading to higher induction of Ft synthesis even if lacking the HO-1 coupled mechanism. This assumption is further strengthened by the observations made here that unlike previous studies with murine systems (see Introduction, section 1.7), the $H_2O_2$ treatment of Jurkat cell lines does not inhibit Ft synthesis but rather induces its synthesis. However, further investigations are needed to clarify the mechanism involved.

**Figure 4.1** summarises the proposed mechanism for differential regulation of labile iron release between the parental and $H_2O_2$ resistant cells following hemin treatment. On the one hand, the results in the parental cells are in agreement with previous observations by Vile and Tyrrell (1993) that iron loading results in the early induction of HO-1

activation as an emergency response to break-down heme and leads to HO-1-dependent increase in Ft and a consequent decreases of the pro-oxidant state of the cell. This contributes to the iron pool that is responsible for the increased Ft synthesis resulting in the decreased levels of intracellular LIP and protection of cell to oxidative damage. On the other hand, our findings in the $H_2O_2$ resistant cells shows, in agreement with previous work by Noel and Tyrrell (1997), that although these cells have higher basal levels of HO-1 as compared to the parental cells, iron loading promotes the early induction of Ft that is not dependent on the induction of HO-1.

In conclusion, the data presented here is the first observation of the direct association between intracellular level of LIP and the resistance of cells to oxidative damage. These findings highlight the crucial importance of the oxidant-induced labile iron release and the increased susceptibility of cells to oxidative damage.

The role of MDR in higher resistance of cells to $H_2O_2$

Further to our investigation on the reasons underlying cellular resistance to $H_2O_2$, an attempt was made to link the high $H_2O_2$ resistance of $H_2O_2$ resistant Jurkat cell line to the development of MDR phenotype in these cells as a result of overexpression of key MDR proteins. Our data indicated no link between MDR and resistance to $H_2O_2$, since both parental and $H_2O_2$ resistant cells appear to have similar drug efflux activity of PgP function and no genes responsible for the MDR phenotype (mdr-1, mrp and mxr) could be detected in either cell line.

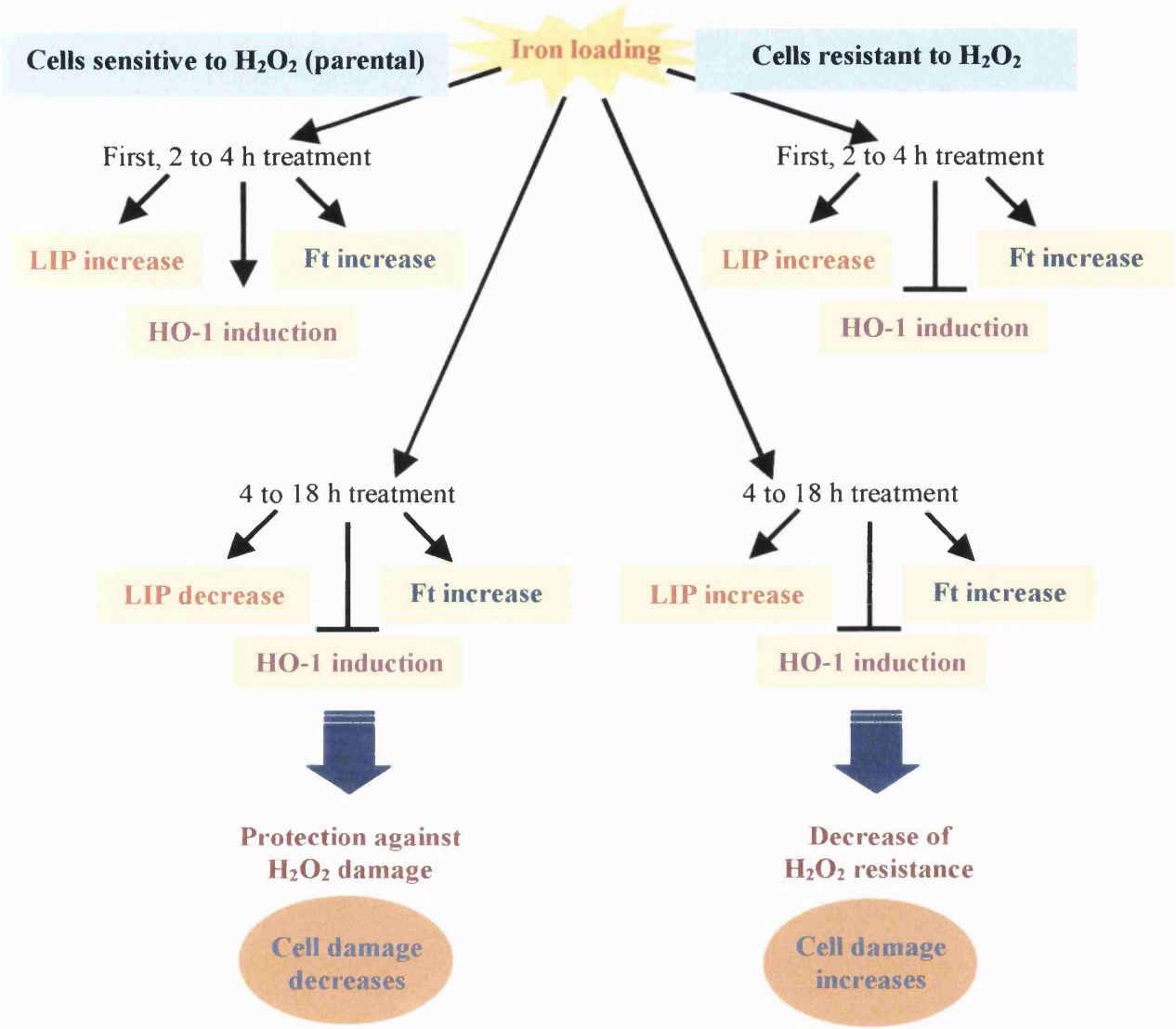



***Figure 4.1*:** A proposed schematic model of the major differential events on parental and $H_2O_2$ resistant cells in respect to their differential sensitivity to oxidative damage.

**The significance of the current study**

The present study highlights the importance of LIP in increased susceptibility of cells to oxidative stress and suggest potential pathways through iron chelation. Our findings also provide new insights into the mechanism of Ft induction by iron loading.

As chemotherapeutic agents eliminate tumor cells by inducing apoptosis in cancer cells via generation of ROS, much effort is now concentrated to elucidate the pathway by which some tumor cells develop antiapoptotic mechanisms to overcome the cell death induced by these agents. Our study should pioneer new approaches to increase the susceptibility of tumor cells to chemotherapy based on modulation of intracellular level of LIP. Furthermore, as higher basal level of Ft, HO-1 and GSH-Px all contribute to higher resistance of $H_2O_2$ resistant Jurkat cells to $H_2O_2$, anti-sense oligonucleotides therapy based on these antioxidant enzymes should provide an efficient mean to render the tumor cell lines more susceptible to chemotherapeutical agents.

**Future projects**

To gain further insight into the different mechanisms of iron regulation under conditions of oxidative stress, we have developed for the first time a cell line resistant to UVA radiation. This newly developed UVA resistant cell line is expected to help us understand to a greater extent the basis of cellular resistance to either UVA- or $H_2O_2$-mediated oxidative stress. Furthermore this cell line should provide further evidence on the correlation between iron and oxidative stress and should therefore help to identify the cascade of events that lead to oxidative damage.

Regarding the MDR studies, we have already developed by clonogenic survival assays, cells resistant to vinblastine (PgP overexpression) and to doxorubicin (MRP overexpression). These cell lines need to be tested for their MDR properties and should be fully characterised in terms of 'basal' and 'induced' level of LIP under conditions of oxidative stress. These newly established cell line might provide more insights into the relation between oxidative stress/iron and MDR.

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